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ATRAZINE AND INFO-DISRUPTION: DOES THE
PESTICIDE ATRAZINE DISRUPT THE TRANSFER OF CHEMICAL
INFORMATION IN THE TERRESTRIAL SALAMANDER,
PLETHODON SHERMANT?

A Thesis
submitted to the Bayer School
of Natural and Environmental Sciences

Duquesne University

In partial fulfillment of the requirement for
the degree of Masters of Science

By
Stephanie Lanzel

August 2008

ATRAZINE AND INFO-DISRUPTION: DOES THE
PESTICIDE ATRAZINE DISRUPT THE TRANSFER OF CHEMICAL
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ABSTRACT

ATRAZINE AND INFO-DISRUPTION: DOES THE
PESTICIDE ATRAZINE DISRUPT THE TRANSFER OF CHEMICAL
INFORMATION IN THE TERRESTRIAL SALAMANDER,
PLETHODON SHERMANI?

By

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August 2008

Thesis Supervised by Sarah K. Woodley

Atrazine is a commonly used herbicide in the United States and is a potential info-disruptor. Atrazine acts as an info-disruptor in fish, by dampening olfactory responses to pheromones, and atrazine has been linked to endocrine disruption in frogs. By interfering with chemoreception, atrazine may potentially impact foraging and reproductive success in various species. I hypothesized that in the terrestrial salamander, *Plethodon shermani*, in which chemical communication is important in social and foraging behaviors; atrazine would act as an odorant and/or interfere with chemosensation. I predicted that both acute and chronic exposure to atrazine would decrease chemoinvestigative behaviors, pheromone detection by the vomeronasal organ (VNO), and pheromone production by tail glands in adult animals.

During acute exposure, where animals were exposed to atrazine for less than 24 hours, there was no change in chemoinvestigation, VNO sensory neuron

activation, or plasma hormone levels relative to controls. Long term atrazine exposure (28 days) did not disrupt chemoinvestigative behavior, pheromone detection by the VNO, pheromone production by the tail glands, or plasma hormone levels. Therefore, although atrazine acts as an info-disruptor in fish, there is no evidence that atrazine impairs the transfer of chemical information in the terrestrial salamander, *P. shermani*.

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TABLE OF CONTENTS

	Page
Abstract.....	iv
Acknowledgements.....	vi
List of Figures.....	x
List of Tables	xi
INTRODUCTION	1
A. Chemosensation	2
B. Atrazine	3
Rationale for thesis work	6
Thesis Objectives	9
Aim 1	9
Aim 2	10
METHODS	11
Specific Aim 1	11
Specific Aim 2	11
General Methods applying to both Aim 1 and Aim 2.....	12
I. Animals.....	12
II. Preparation of Atrazine	13
III. Verification of Atrazine Exposure Concentrations.....	14
IV. Preparation of Chemosensory Cues.....	15
V. Behavioral tests.....	17
A. General methods.....	17
B. Nose tapping and locomotor activity.....	17
C. Discrimination of atrazine versus water	19

V. Vomeronasal responses to chemosensory cues.....	20
A. Method of Agmatine (AGB) uptake	20
B. Testing responsiveness of the VNO	20
C. Tissue processing following AGB uptake.....	21
D. AGB immunocytochemistry	21
E. Image analysis of AGB-IR in the VNO.....	22
VII. Hormone Measurement	23
VIII. Statistics	23
Methods specific to Aim 1	24
I. Nares Occlusion.....	24
A. Blockage of external nares	25
Methods specific to Aim 2.....	25
I. Long term exposure to atrazine contaminated substrates.....	25
A. Weekly monitoring.....	26
B. Feeding studies	26
II. Tail Gland Morphology.....	26
A. Tissue processing	26
B. Image analysis	27
RESULTS	28
I. Verification of Atrazine Exposure Concentrations	28
Aim I.....	28
I. Behavior.....	28
A. Nose-tapping response to chemosensory cues.....	28
B. Discrimination of atrazine versus water.....	30
II. Agmatine uptake after exposure to chemosensory stimuli.....	31

III. Hormone levels in response to atrazine exposure.....	32
Aim II.....	32
I. Behavior.....	32
A. Body Mass.....	32
B. Feeding.....	32
C. Nose-tapping response to chemosensory cues	33
II. Agmatine uptake in response to chemosensory stimuli	33
III. Tail Gland Morphology	34
IV. Hormone levels	34
DISCUSSION	35
Chemoinvestigation during acute atrazine exposure	35
Vomeronasal activation following acute atrazine exposure	37
Plasma hormone response following acute atrazine exposure.....	39
Behavioral responses to long term atrazine exposure.....	40
Vomeronasal response following long term atrazine exposure	40
Tail gland responses to long term atrazine exposure	41
Plasma hormone levels in response to long term atrazine exposure.....	41
Conclusions.....	42
FIGURES	45
REFERENCES	66

LIST OF FIGURES

	Page
Figure 1. Nose tapping on different chemosensory cues with or without atrazine	45
Figure 2. Activity on different chemosensory cues with or without atrazine	46
Figure 3. Nose tapping on different doses of atrazine	47
Figure 4. Nose tapping on different doses of atrazine with non-exposed control group	48
Figure 5. Nose tapping on prey cue and social cue with or without atrazine	49
Figure 6. Nose tapping in response to nares occlusion.....	50
Figure 7. Discrimination of atrazine versus water	51
Figure 8. Photopictomicrographs of VNO sections with AGB-IR	52
Figure 9. AGB-IR in response to atrazine and social cue.....	53
Figure 10. AGB-IR in response to atrazine and prey cue	54
Figure 11. AGB-IR in response to prey cue and social cue.....	55
Figure 12. Nose tapping following long term atrazine exposure.....	56
Figure 13. Photopictomicrographs of VNO sections with AGB-IR	57
Figure 14. AGB-IR following long term atrazine exposure	58
Figure 15. Tail gland morphology following long term atrazine exposure	59
Figure 16. Photopictomicrographs of tail gland morphology	60

LIST OF TABLES

	Page
Table 1. Atrazine concentrations during short term exposure	61
Table 2. Atrazine concentrations during long term exposure	62
Table 3. Plasma corticosterone levels following acute exposure to prey cue and/or atrazine.....	63
Table 4. Plasma corticosterone and testosterone levels following acute exposure to social cue and/or atrazine.....	64
Table 5. Plasma corticosterone levels following chronic exposure to atrazine ...	65

INTRODUCTION

Non-lethal concentrations of various chemicals interrupt the transfer of chemical information within and between species and are classified as ‘info-disruptors’ (Lürling and Scheffer, 2007). Info-disruptors are a new category of chemical risks that can have adverse affects on organisms through more subtle or indirect mechanisms than those typically assessed in toxicological studies. Like endocrine disruptors, ‘info-disruptors’ could have widespread ecosystem impacts. Info-disruptors can disrupt the ability to detect or respond to chemosensory stimuli, as well as disrupt the ability to produce chemosensory stimuli, such as pheromones.

Vital information transfer can be altered by pollutants such as pesticides, heavy metals, surfactants and stabilizers, and acidification (Lürling and Scheffer, 2007). Disruption has been noted at low, ecologically relevant concentrations and resulted in altered chemical cue production and detection in a wide variety of organisms (Cheek et al., 1998; Lürling and Scheffer, 2007). Info-disruption affects interactions between organisms, which in turn could have large-scale effects on populations and communities. For example, heavy metals induced anti-predator reactions in frogs (Lefcort et al., 2000), low levels of the pesticide diazinon muted the response of Chinook salmon to alarm secretions (Scholz et al., 2000), and mud snails did not respond to gender-specific pheromones following exposure to organotin compounds (Straw and Rittschof, 2004). These varied examples illustrate the multitude of mechanistic effects of pollutant exposure on information transfer via chemical communication.

A. Chemosensation

Pheromones are chemical signals that are secreted into the environment by one organism to communicate with another organism of the same species, which elicit a change in behavior and/or physiology. These chemical signals are effective at low concentrations and can influence the endocrine and reproductive systems (Johnston, 2000). Some pheromones relay information about gender and reproductive state; others may act as alarm signals to indicate the presence of a predator (Palmer, 2004; Thompson et al., 1999). Atrazine and other synthetic chemicals can disrupt normal cell signaling and may reduce an organism's ability to respond to chemosensory stimuli, such as pheromones (Lüring and Scheffer, 2007). Atrazine can act to influence both the production and detection of relevant chemical cues in the environment.

Atrazine may also interfere with the ability of organisms to respond to chemosensory cues emitted by other species. For example, many animals rely on the detection of chemosensory cues from prey when foraging. Vertebrates detect chemical cues via the olfactory system, comprised of both the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Chemosensory cues can be detected by sensory neurons in both the MOE and VNO, but volatile odors are primarily detected by the ciliated epithelium of the MOE, while non-volatile odors are detected by the microvillar receptors of the VNO (Halpern, 1987; Johnston 2000). In addition, the VNO detects many pheromones. Neurons of the VNO project into the accessory olfactory bulb of the brain. From there, the chemosensory pathway continues to the medial amygdala and the hypothalamus,

where reproductive behaviors and reproductive endocrine responses to a signal are processed (Halpern, 1987; Scalia and Winans, 1975; Schmidt and Roth, 1990).

B. Atrazine

Atrazine is a commonly used herbicide in the United States and is a potential endo-disruptor in vertebrates (Moore and Lower, 2001; Moore and Waring, 1998, Saglio and Trijasse, 1998, Rohr et al., 2006). It is used primarily in corn and sorghum production and has been a major agricultural herbicide for over 50 years. Additionally, it is applied in forestry and turf maintenance. Atrazine is highly mobile in both terrestrial and aquatic ecosystems. It is resistant to degradation, with a reported half-life of 95-350 days (Diana et al., 2000), and is found 10 to 20 times more often in water quality monitoring studies than the second most detected pesticide (Ribaud and Bouzaher, 1994). Atrazine degrades fairly slowly (1-2 years) once in the water column (Ribaud and Bouzaher, 1994). Currently atrazine is listed as a restricted use pesticide (RUP) (US EPA, 2003) and maximum contaminant levels are set at 3 ppb for drinking water and 300 ppb for surface waters. Atrazine formulations can be sprayed to crops at concentrations as high as 213 g/L (213,000,000 ppb) and have been detected in natural waters at 12,700 ppb (Lenkowski et al., 2008). As a restricted use pesticide, atrazine must be continually assessed to remain registered under the EPA, with assessments focusing on watershed quality, drinking water contamination, human health, and wildlife impacts.

Atrazine has been shown to act as an info-disruptor by interfering with olfactory-related physiological processes in many species (Rohr and Crumrine, 2005; Moore and Waring, 1998; Hayes et al., 2002). In salmon, short-term exposure to atrazine (5 days) resulted in a reduced olfactory epithelial response in males to sex pheromones released by the female, and reduced plasma levels of several sex steroids including testosterone, 11-ketotestosterone, and 17,20 β -dihydroxy-4-pregnen-3-one (Moore and Lower, 2001; Moore and Waring, 1998). By reducing the ability of males to detect female sex pheromones, atrazine could reduce overall species reproductive success if males are unable to find mates.

Other chemicals have also been shown to interfere with pheromone production. Studies in the male newt *Notophthalmus viridescens* found that low doses of the insecticide, endosulfan, reduced the size of pheromone secreting glands in the tail (Park and Propper, 2002). Additionally, males expressed reduced latency times in response to female odors from females exposed to endosulfan, indicating that these females had reduced pheromone production following exposure. This reduction in olfactory responsiveness by males towards females may result in reduced mating success (Park and Propper, 2002; Park et al., 2001).

In addition to influencing the detection of social cues, atrazine influences the expression of behaviors that are triggered by chemosensory cues. Short-term exposure (24 hour) to atrazine or diuron induced changes in anti-predator behaviors of juvenile goldfish, such as burst swimming. Burst swimming is triggered by olfactory detection of alarm pheromones. Goldfish increased stress

response activities and alarm reactions after acute exposure to atrazine (Saglio and Trijasse, 1998). In terrestrial ambystomatid salamanders, atrazine increased motor function (skittishness) after a 37 day exposure, possibly a result of the disturbance of the nervous system (Rohr et al., 2003).

Perhaps the most well known study of the effects of atrazine on wildlife is the work of Tyrone Hayes on the feminization of male frogs. Initial studies reported a demasculinization of the larynx in male *Xenopus laevis* larvae following atrazine exposure at 1 ppb. Hayes concluded that atrazine was acting as an endocrine disruptor, by converting androgens to estrogens, thereby reducing the androgen-dependent growth of the larynx (Hayes, 2004). To further examine if males were 'chemically castrated' when exposed to atrazine, Hayes examined the impact of atrazine exposure during development on gonadal differentiation in *Rana pipiens*, and found that exposure to atrazine led to development of testicular oocytes and retarded gonadal development at low doses (0.1 and 25 ppb). These laboratory findings were supported by additional work in the field where multiple sites in agricultural regions were sampled to examine reproductive organs in male frogs. Results showed a link between atrazine exposure and hermaphroditism (Hayes et al., 2002). In a subsequent study, Hayes et al. (2006) examined the effect of a mixture of nine pesticides on plasma corticosterone levels in the male African clawed frog, *X. laevis*. They found a four-fold increase in circulating levels of corticosterone in pesticide-exposed animals when compared to control males (Hayes et al., 2006). Corticosterone is a metabolic and stress hormone with

multiple effects on the body. Increased corticosterone due to atrazine exposure could have deleterious effects (Hayes et al., 2006).

Rationale for thesis work

Populations of amphibians have been declining worldwide. Pesticides may be a factor due to widespread use and high levels of runoff. Amphibians have permeable skin used for gas exchange, which makes them susceptible to chemicals in the environment. Most studies have focused on the impact of short term exposure to atrazine, and less is known about chronic effects of atrazine exposure.

The aim of my work was to examine effects of both acute and chronic atrazine exposure as a potential info-disruptor in an amphibian, the plethodontid salamander, *Plethodon shermani*. Due to the widespread use of atrazine in the United States and its persistence in many environments, it is reasonable to assume that many plethodontid populations will be exposed to the pesticide. Plethodontid salamanders are especially vulnerable to environmental toxins because they lack lungs and all gas exchange occurs through their skin. They are an ideal system to study info-disruption because chemical communication is used in mating, territoriality, prey detection, and predator avoidance (Houck, 1998; Houck and Reagan, 1990; Houck et al., 1998; Schubert et al., 2006, Schubert et al., 2008; Jaeger and Gergits, 1979; Placyk and Graves, 2002). In addition, plethodontid salamanders are widespread and the most abundant vertebrates in many

ecosystems; including many environments where atrazine may be found (Burton et al., 1975; Petranka et al., 2001).

My goal was to examine whether atrazine impacted chemical communication. Although info-disrupting effects of atrazine on chemical communication may be subtle and sublethal, they may alter an organism's ability to detect biologically relevant social cues and/or prey cues. If atrazine is acting as an info-disruptor in *P. shermani*, it may alter the organism's ability to detect important sensory cues. First, I assessed whether short-term exposure to atrazine-contaminated substrates dampened or eliminated chemoinvestigation. Studies determined whether an animal could smell atrazine and whether atrazine reduced chemoinvestigation of biologically relevant chemical cues, if atrazine was presented in combination with a chemical cue. Initial testing used a high dose of atrazine to increase the likelihood of eliciting a response. Subsequent studies examined a range of doses of atrazine. I also examined whether acute atrazine exposure interfered with the ability of sensory neurons of the vomeronasal organ to detect pheromones and other chemical cues. Finally, I measured plasma corticosterone levels, because a previous study indicated that atrazine may contribute to increased plasma corticosterone (Hayes et al., 2006).

In the field, animals may be chronically exposed to environmental toxins. Animals were exposed to atrazine for several weeks (28 days) to determine whether chronic exposure to atrazine interfered with detection of chemical cues, feeding, pheromone production, or plasma corticosterone levels. First, feeding studies were included because plethodontid salamanders use chemical cues to

locate prey items (Placyk and Graves, 2002) and previous studies in crayfish, goldfish, and insects found that sublethal concentrations of synthetic chemicals interfered with chemoreception of prey cues (Lürling and Scheffer, 2007). Secondly, I examined whether long-term exposure to atrazine interfered with the ability of sensory neurons of the vomeronasal organ to detect pheromones and other chemical cues that are detected by the vomeronasal organ. Next, pheromone production was examined because studies in the male newt *N. viridescens* showed that the insecticide, endosulfan, reduced the size of pheromone secreting glands in the tail (Park and Propper, 2002). Thus, I examined tail gland size in *P. shermani* because previous studies indicated that the tail was an important source of skin secretions related to scent marking of territories as well as related to anti-predator defenses (Hecker et al., 2003; Lagen and Woodley, submitted). Finally, plasma corticosterone levels were measured to determine if long term atrazine exposure increased circulating levels.

Thesis Objectives

Aim 1—Effects of acute atrazine exposure on chemoinvestigation and vomeronasal organ (VNO) sensory neuronal activation.

With this aim, I sought to determine whether atrazine is detected by *Plethodon shermani*, and/or if acute exposure interferes with detection of chemical cues (prey cues, alarm secretions, or courtship pheromones) as indicated by changes in chemoinvestigation or changes in activation of vomeronasal organ sensory neurons. I conducted experiments to test the following hypotheses and predictions:

H₁: Atrazine is acting as an odorant and is detected by chemosensory neurons of the nasal cavity.

I predict that animals exposed to atrazine will chemoinvestigate substances moistened with atrazine more than substrates moistened with water. I also predict that blocking the nares of animals will eliminate the ability of odorants to enter the nasal cavity, which will eliminate any potential nose-tapping response to atrazine. Finally, I predict that atrazine will activate sensory neurons of the VNO using the methods of agmatine uptake.

H₂: Acute exposure to atrazine will decrease the ability of *P. shermani* to detect chemosensory cues.

I predict that atrazine exposure will result in fewer nose taps (a chemoinvestigatory behavior) in response to chemosensory cues compared to animals exposed to water. I also predict that exposure to atrazine will reduce activation of VNO sensory neurons by other chemosensory cues using the method of agmatine uptake.

Aim 2—Effects of long-term atrazine exposure on chemoinvestigation, vomeronasal organ sensory neuron activation, feeding, plasma hormone levels, and tail gland morphology.

With this aim, I examined whether chronic exposure to atrazine results in info-disruption. I conducted experiments to test the following hypothesis and prediction.

H₃: Chronic exposure to atrazine interferes with detection and production of chemical cues.

I predict that 28 days of exposure to atrazine-contaminated substrates will decrease feeding, decrease nose tapping behavior, reduce responsiveness of the VNO to chemosensory stimuli, and alter the morphology of pheromone-producing tail glands.

METHODS

Specific Aim 1

In a series of experiments, animals were acutely exposed (1 hour) to atrazine contaminated substrates, during which time nose tapping behavior in response to various chemosensory cues was measured. Since nose tapping correlates with activity, I also measured locomotor activity. Initially, high doses of atrazine were used, and follow-up studies examined the effect of a range of atrazine concentrations on behavior (nose tapping, locomotor activity, avoidance behavior).

To further investigate whether animals could detect atrazine and whether it could be detected through the olfactory system, the nares of animals were blocked to prevent access of chemosensory cues to the nasal cavity. Levels of chemosensory investigation in response to various chemosensory cues were measured in nares occluded and non-occluded animals.

Following behavioral studies, chemical cues were applied to the nares in conjunction with agmatine (a marker of VNO sensory neuron activation) to measure the response of VNO sensory neurons to acute atrazine exposure. In some cases, animals were simultaneously exposed to atrazine and a chemosensory cue.

Specific Aim 2:

Animals were exposed to atrazine (0 ppb or 300 ppb) over a four week period and monitored throughout that time to assess feeding and changes in body

mass. Following 4 weeks of atrazine exposure, levels of chemoinvestigation in response to different chemosensory cues were measured over a one-week period, during which the animals continued to be housed on atrazine-contaminated substrates.

Following nose-tapping behavioral studies, the ability of the VNO to respond to chemosensory cues was measured using agmatine (AGB) uptake. Finally, tail sections were examined to determine the effects of atrazine on the production of pheromones by the cutaneous tail glands.

General Methods applying to both Aim 1 and Aim 2

I. Animals

Methods were approved by Duquesne University's Institutional Animal Care and Use Committee (IACUC). Animals were collected from a single location in North Carolina (Wayah Bald, Macon County, NC, 83° 30' 30"N longitude; 35° 10' 49"W latitude) with appropriate permits from the Department of Wildlife in August 2005 [short term atrazine exposure], August 2006 [long term atrazine exposure] and August 2007 [nares occlusion study]. Males used in the succeeding studies had their mental glands surgically removed following capture. Animals were housed at Oregon State University where they participated in mating behavioral experiments and were shipped to Duquesne University in May 2006 (August 2005 caught), May 2007 (August 2006 caught), or January 2008 (August 2007 caught) and were housed individually at 16°C on a 14 hour

light : 10 hour dark photoperiod in 16 x 16 x 5 cm plastic boxes lined with moist brown paper towels. Animals were fed wax worm larvae every 2-3 weeks.

Sex and reproductive condition were determined by examining the ventral abdominal body wall for the presence of testes (males), presence of ova (reproductive/gravid females), or absence of ova (non-reproductive/non-gravid females) and later confirmed with dissections. Size was also used in sex determination; males are slightly smaller in average body length than females. Body length was determined by measuring the distance from snout to the anterior end of the cloacal vent.

II. Preparation of Atrazine

In my studies, a high dose of atrazine was used initially to increase the likelihood of finding a response. High doses of atrazine were modeled after Jason Rohr's studies using a high dose of atrazine at 400 ppb (Rohr et al., 2003; Rohr and Palmer, 2004; Rohr et al., 2004). Subsequent dose-response studies examined effects of lower doses. These lower doses were used to expose animals to the range of contamination levels found in the field. Currently, the U.S. Environmental Protection Agency has set ambient surface water standards at 300 µg/L (ppb) for acute exposure and 12 µg/L (ppb) for more chronic exposure (U.S. EPA 2001).

In the first behavior studies (short term atrazine exposure, dose-response, simultaneous choice), atrazine stock solution was prepared by dissolving 3 mg of atrazine in approximately 3.5 ml of acetone and then diluting in 1L nanopure

water to create a stock solution of 3,000 ppb. Because we were concerned that animals might be responding to the acetone used to dissolve the atrazine, subsequent studies prepared a stock solution of 30,000 ppb by dissolving 30 mg atrazine in 1L nanopure water without the addition of acetone. All solutions were mixed on a stir plate overnight until dissolved. Concentrations of atrazine stock solutions were verified using Beacon Analytical Test Kits. Stock solutions were diluted in order to run the atrazine assay, which had a standard curve that measured from 0.05 ppb to 5.0 ppb atrazine. Atrazine stock solution was stored in glass containers at -20°C in darkness to prevent photodegradation. Stock solutions were used for no longer than 3 months and were diluted to necessary concentrations for testing. Water controls were prepared, stored, and diluted following the same methods.

III. Verification of Atrazine Exposure Concentrations

It is possible that atrazine was binding to the plastic or towel substrates in some of the experiments and therefore might not have been available to the animals. In short term behavioral assays, animals were placed for 1 hour in a bioassay tray with a paper towel moistened with 700 ppb of atrazine. In order to verify the concentration of atrazine animals were actually exposed to, bioassay trays with a paper towel moistened with 15 ml of 700 ppb of atrazine were set up for 1 hour. As a control, 15 ml of 700 ppb of atrazine were added to the bioassay trays without any towels. After 1 hour, the liquid was poured off of each tray and was assayed for atrazine content.

In the long term study, animals were housed in home boxes lined with towels moistened with 300 ppb of atrazine for 4 weeks, during which the papers were replaced every week. In order to verify the concentration of atrazine animals were actually exposed to, home boxes lined with towels moistened with 45 ml of 300 ppb of atrazine were set up for 1 week. As a control, 45 ml of 300 ppb of atrazine were added to home boxes and set up without any towels. After 1 week, excess liquid was poured off and assayed for atrazine content. Stock solution of the desired concentration was included in the assay as an additional control.

IV. Preparation of Chemosensory Cues

I was interested in whether atrazine disrupted detection of a variety of chemosensory stimuli. Both behavioral and VNO sensory neuron responses to several different chemosensory cues were examined depending on the particular study. Below I describe the different chemosensory cues I used.

Female skin secretions and female body rinses increase chemoinvestigation and are detected by sensory neurons of the VNO (Schubert et al., 2008; Schubert et al., 2006; Schubert and Woodley, unpublished data). Skin secretions were prepared by placing an animal in 100 ml of ddH₂O for 5 minutes while gently pressing the tail of the salamander with blunt forceps. This procedure induces the release of copious secretions that may function to deter predators (Evans and Brodie, 1994; Graves and Quinn, 2000). Female body rinse was obtained by placing a gravid female animal into 50 ml of ddH₂O in round

glass jar for 48 hours at 16°C on a 14L:10D photoperiod. Alarm secretions and body rinses were pooled separately, frozen in aliquots, and used at full strength within 7 days of collection.

Prey cues were used to evaluate the effect of atrazine on the ability to gather information for feeding. I initially examined responses to earthworm wash. Earthworms are prey items for *P. shermani* in the wild, they will eat earthworms in the laboratory, and there was some evidence that earth worm wash activated sensory neurons of the VNO (Maddamma and Woodley, unpublished). Also, earthworm wash has been well studied in garter snakes, where it elicits both a behavioral (tongue-flicking) and a VNO response (Jiang et al, 1990; Wang et al, 1993). Earthworm wash was made using previously established methods (Halpern et al., 1984; Halpern et al., 1993) from earthworms obtained from Highlands Biological Station collected in August 2006. After rinsing with ddH₂O, earthworms were heated (in a ratio of 6 g of worms/20 ml ddH₂O) to 60°C while stirring gently with forceps for 1 minute. Worms were discarded and the solution was centrifuged. The supernatant was removed and stored at -20°C.

Because I was unable to discern behavioral or VNO neuron responses in *P. shermani* to earthworm wash in my studies, subsequent studies used wax worm macerate. Animals are normally fed wax worms in the laboratory. To create the cue, wax worms were rinsed to remove any food particulate and macerated in a glass beaker to create a worm paste. The ratio of 6 g of worms/20 ml ddH₂O was modeled from the earthworm wash. The solution was centrifuged at 30,000 RPM and 25°C for 45 minutes. The supernatant was removed and spun again at 40,000

RPM and 20°C for 45 minutes. The supernatant was diluted to 20% in ddH₂O and stored at -20°C. Prey cues were used within 7 days of collection.

Mental gland extract was used to test responsiveness of the VNO to chemosensory stimuli. During courtship, males contact their mental gland to the nares of females. The pheromones present in mental gland decrease duration of courtship and increase receptivity of the female (Houck et al., 1998) and have been shown to activate VNO sensory neurons (Schubert et al., 2008; Schubert et al., 2006; Wirsig-Wiechmann et al., 2002). Mental gland extract was a gift from Dr. Richard Feldhoff (see Schubert et al., 2006 for details on preparation). Mental gland extract was used at a concentration of 2.0 mg/ml which was previously demonstrated to elicit increased activation in VNO cells (Wirsig-Wiechmann et al., 2002, Wirsig-Wiechmann et al., 2006, Schubert et al., 2006, 2008).

V. Behavioral tests

A. General methods

Scan sampling methods (Martin and Bateson, 1993) were used to score behavioral responses to chemosensory stimuli in multiple subjects at one time. All behavior tests were conducted at 25°C under dim incandescent light in the evening hours during the dark period of the photoperiod, to mimic nocturnal conditions. One investigator performed all tests and was blind to treatment groups when quantifying behavior.

B. Nose tapping and locomotor activity

In my studies, I focused on a chemoinvestigatory behavior called nose tapping, a behavior that transfers non-volatile chemical cues from the substrate into the lumen of the vomeronasal organ (VNO), a specialized region of the olfactory system that detects pheromonal cues. The neurons of the VNO project to the accessory olfactory bulb of the brain. From the accessory olfactory bulb, information is sent to the medial amygdala and the hypothalamus, where behavioral and physiological responses to the chemical cue are regulated (Halpern, 1987; Scalia and Winans, 1975; Schmidt and Roth, 1990). Animals touch the tip of the nare to a substrate in the environment to sample for chemosensory information. Levels of nose tapping may be correlated with locomotor activity; therefore, the movement of each animal on the substrate was also monitored.

Nose tapping and activity were measured by placing each animal in a 23 x 23 x 2 cm plastic testing chamber lined with brown paper towels moistened with 10 ml of a particular chemosensory cue. Using scan sampling, the presence or absence of nose tapping behavior was scored once every minute for 60 minutes (1 = nose tapping observed; 0 = no nose tapping observed). Location (in one of 4 possible quadrants within the testing chamber) was also noted to assess activity. The final score was defined as the total number of scans where nose tapping behavior was observed (maximum possible of 60) and the number of times the animal was located in a quadrant that was different from the quadrant in the previous scan (maximum possible of 60).

Using a repeated measures design, animals were tested on substrates moistened with water, non-gravid female alarm secretion, gravid female body rinse, wax worm macerate, and/or atrazine, depending on the experiment. Each animal was exposed to a single chemosensory cue on a given night. In the acute atrazine behavior studies, animals were tested every fourth night with a single type of chemosensory cue per night. In the nares occlusion study and long term behavior studies, subjects were tested every other night. The investigator was blind to the identity of the chemosensory cue. The order of the chemosensory cues was randomized across each animal.

C. Discrimination of atrazine versus water

Each animal was placed in a 23 x 23 x 2 cm plastic testing chamber. One half of the chamber was lined with a brown paper towel moistened with water and the other half lined with a brown paper towel moistened with atrazine. A 1 cm gap was left between the two towels to prevent transfer of cues between the two substrates.

Using scan sampling, the location of each animal's head was noted once every two minutes for two hours. Animals that spent more than 50% of the 60 observations on the side moistened with water were considered to show an avoidance of atrazine. Following the two hour observation period, animals were left in testing chambers overnight and the animal's location the next day was recorded twelve hours later. This location is an indicator of where the animal will likely spend the rest of the day, as activity decreases during daylight hours.

Animals with heads positioned on the water were considered to show an avoidance of atrazine.

V. Vomeronasal responses to chemosensory cues

A. Method of Agmatine (AGB) uptake

The ability of the VNO to respond to chemosensory cues was measured using the method of agmatine (AGB) uptake. Agmatine is a modified amino acid that passes through open cation channels. The method of AGB uptake provides an index of VNO sensory neuron activation. Agmatine was administered in conjunction with a chemosensory cue. Those cells that were activated by the chemosensory cue had opened ion channels, where AGB could enter non-specifically. The sensory neurons that were stimulated by the chemosensory cues would contain the AGB, which can be visualized using standard immunohistochemical techniques. This method has been used in *P. shermani* to demonstrate that male courtship pheromones and female skin secretions are detected by the VNO (Wirsig-Wiechmann et al., 2002, Schubert et al., 2006; Schubert et al., 2008).

B. Testing responsiveness of the VNO

Immediately prior to application, chemosensory cues were individually mixed 1:1 with 6mM AGB dissolved in 0.1M PBS following the methods of Wirsig-Wiechmann (2002). Control animals received water + AGB. Animals were placed individually in clean home boxes and 2 μ l of the chemosensory stimulus was delivered to the anterior tip of the external nares every 2 minutes for

44 minutes, yielding 22 applications. Phosphate buffered saline was used as a wash following AGB application at 3 x 5µl. The saline was applied to rinse any residual AGB from the lumen of the nasal cavity. Animals were processed in two batches by two investigators.

C. Tissue processing following AGB uptake

Immediately following exposure to agmatine in conjunction with chemosensory cues, animals were sacrificed via rapid decapitation. Trunk blood was collected to determine plasma hormone levels. Following decapitation, the lower jaw was removed from the head and heads were fixed in a 4% paraformaldehyde, 2.5% glutaraldehyde PBS solution, pH 7.4 overnight. The next day tissues were decalcified using DeCal (DeCal Corporation, Congers, NY) for three days then cryoprotected in 30% sucrose/PBS for 2 days. Tissues were then placed in a 1:2 solution by volume of 30% sucrose/PBS and OCT embedding matrix (Fisher Scientific) for 1 day. Heads were removed from the sucrose/OCT solution, rinsed in ddH₂O, and embedded in OCT in blocks containing 4-5 heads randomly assigned across treatment groups. The blocks were frozen and stored at -20°C until they were sectioned using a cryostat. Sections were cut coronally at 20µm at -14°C. Sections containing VNO tissue were collected on poly-lysine coated superfrost slides and allowed to dry at 37°C for 1 hour. Slides were stored at -80°C and immunocytochemistry for AGB was performed within 3 weeks.

D. AGB immunocytochemistry

Standard immunocytochemistry methods were used to identify cells immunoreactive for AGB. Every 4th section underwent immunocytochemistry for

AGB. The average diameter of an AGB-immunoreactive VNO cell body is 8 μ m (Schubert and Woodley, unpublished data), so processing every 4th section prevents counting a single cell more than once. Tissues were rinsed 6 x 5 minutes with 0.1M PBS then incubated for 30 minutes in a preincubation solution of 0.2% Triton-X, 1% normal goat serum, and 0.004% sodium azide in PBS. Tissues were incubated lying flat in rabbit polyclonal anti-AGB antibody (Chemicon) diluted 1:4000 in preincubation solution for 3 days in a humid chamber at room temperature. Tissues were rinsed 6 x 5 minutes in PBS, incubated 30 minutes with biotinylated goat anti-rabbit antibody, rinsed in PBS, incubated for 30 minutes with Avidin-Biotin Enzyme Complex (ABC elite, Vector) and rinsed in PBS. Tissues were rinsed once in 0.05 M Tris-HCL, pH 7.4, incubated for approximately 5 minutes in 0.02% DAB-0.001% H₂O₂ and rinsed 5 x 5 minutes in Tris-HCL. Slides were coated with Crystal Mount (Biomedica) and allowed to dry overnight or dehydrated in EtOH and coverslipped with Permount (Fisher Scientific).

E. Image analysis of AGB-IR in the VNO

Slides were coded so the investigator was blind to treatment. All tissues were examined using an Olympus brightfield microscope to count all AGB-IR cells, identified by darkly stained cytoplasm. Every 4th section was counted on both the left and right nasal cavities for the entire length of the VNO. The total number of AGB-IR cells was summed per animal.

VII. Hormone Measurement

Plasma steroid hormone levels were measured in trunk blood collected from each animal within 3 minutes of sacrifice. Samples were centrifuged and plasma was frozen at -20°C until assayed. All plasma hormone levels were measured by the Endocrine Services Laboratory at the Oregon Primate Research Center.

VIII. Statistics

Statistical analyses were completed using SPSS for Windows (Release 12.0.0. 2003. Chicago: SPSS, Inc.)

In Aim 1, chemoinvestigation and activity were analyzed using a repeated measures ANOVA with substrate (chemical cue) as the within-subjects factor. Significant main effects were followed up with within-subjects contrasts. In Aim 2, chemoinvestigation and activity were analyzed using a 2-way repeated measures ANOVA with substrate (chemical cue) as the within-subjects factor and treatment group (atrazine exposure concentration, 0 ppb vs. 300 ppb) as the between-subjects factor. To examine the role of locomotor activity in chemoinvestigation, I conducted a 1-way ANOVA for each chemosensory cue with group as the between-subjects factor and with activity as a covariate. Because locomotor activity was not a significant covariate, it was not included in analyses of chemoinvestigation.

Tests for discrimination between atrazine and water were analyzed using 2-tailed binomial tests.

In Aim 1, the number of AGB-IR cells was analyzed with a 2-way ANOVA with chemosensory cue and atrazine exposure as the between-subjects factors. In Aim 2, the number of AGB-IR cells was analyzed with a 2-way ANOVA with chemosensory cue and group (atrazine exposure concentration) as between-subjects factors. Significant main results were further analyzed using SNK post-hoc tests for pair-wise comparison.

Plasma hormone levels were analyzed using a 2-way ANOVA with chemical cue and atrazine exposure as the between-subjects factors in Aim 1. In Aim 2, plasma hormone levels were analyzed using a 1-way ANOVA with atrazine exposure as the between-subjects factor.

In Aim 2, the dermal thickness of the tail was analyzed with a 1-way ANOVA with atrazine exposure group as the between-subjects factor.

In all statistical tests using parametric statistics, data were normally distributed and variances were homogeneous. In some cases, data were log transformed before analysis in order to satisfy assumptions of parametric statistics.

Methods specific to Aim 1

I. Nares Occlusion

To determine if atrazine was detected by sensory neurons of the main olfactory epithelium, inert silicon gel was applied to the nares of animals to seal off the olfactory system. Animals were anesthetized and the inert silicon gel was

applied to the nares (experimental) or top of the head (control). This method was shown previously to block the nares of newts (Ferrer and Zimmer, 2007).

A. Blockage of external nares

All animals were sedated using 2-3 drops of baby Orajel, applied to the dorsal surface along the vertebral column, to reduce movement. The external nares of an animal were blocked by applying inert silicon gel (Corning vacuum grease, Dow Corning, Midland, MI) to the external openings with a cotton swab following the methods of Ferrer and Zimmer (2007). Following application, animals were returned to their home boxes and given 12 hours to recover. Silicon gel remains on the nares for 24-48 hours; therefore application occurred 12 hours prior to starting monitoring of nose tapping behaviors. [Pilot studies showed that Orajel anesthesia did not influence locomotor activity when behavior was tested 12 hours after application.]

No long-term damage to epithelial cells has been noted following application of the inert silicon gel (Ferrer, pers. comm.). Control males were treated in the same manner except that silicon gel was applied to the top of the head. Animals were each tested in a repeated measures design on substrates moistened with several different chemosensory cues.

Methods specific to Aim 2

I. Long term exposure to atrazine contaminated substrates

Animals were housed individually at 16°C on a 14L:10D photoperiod in 16 x 16 x 5 cm plastic boxes lined with moist brown paper towels with crumpled

paper towels as cover items. Towels were moistened with 35 ml of water or of atrazine: substrate papers (2 folded paper towels) were moistened with 15 ml of solution and each cover item (2/box) was moistened with 10 ml of solution. Towels were replaced weekly with fresh towels to minimize degradation of atrazine (half life in water = 86-180 days; Seybold et al., 2000; Agency for Toxic Substances, 2003).

A. Weekly monitoring

Each week, when paper towels were changed, body mass was measured.

B. Feeding studies

In long-term atrazine exposure studies, feeding was monitored weekly.

Prior to the study, animals were fed *Drosophila melanogaster* to acclimate subjects to the flies. During the study, each subject was given 15 frozen *Drosophila* in the evening hours in their home boxes with cover items removed. The next morning the number of *Drosophila* remaining in the box was counted. Animals were tested with *Drosophila* once weekly for three weeks; the first after one full week of exposure to atrazine substrates.

II. Tail Gland Morphology

A. Tissue processing

Sections of the rostral tail were processed to examine thickness of the skin. Following sacrifice, intact tails were placed in 10% neutral buffered formalin for 24 hours and then rinsed in ddH₂O for 24 hours. A 1.5 cm tail segment rostral to the cloaca was placed in decalcification solution (DeCal, Decal

Corporation) for 4 days, 30% sucrose/PBS for 2 days and 2:1 sucrose:OCT for 1 day. Following processing, tail segments were embedded in a matrix of OCT in blocks to be sectioned using a cryostat. Ten animals were randomly selected from each treatment group. Tails were sectioned at -18°C at 30 µm. Every 10th section was collected on poly-lysine coated superfrost slides (for a total of 300 µm of tail collected) and allowed to dry at 37°C for 1 hour. Slides were stored at room temperature until stained using Hematoxylin and Eosin using standard methods. Following staining, cover-slips were affixed with permount.

B. Image analysis

For each animal, 8-10 sections were randomly chosen for analysis. Sections were distributed evenly down the length of the rostral tail segment. Images were captured using an Olympus DP70 digital camera and the cross sectional area of the tail section was measured using Image-Pro Plus software. The dermal thickness was measured in three locations adjacent to the fat deposits found in the tail. One measurement was collected from the center of the fat deposit and one measurement was collected from each of the two sides measuring height vertically. These measurements were made in both the dorsal and ventral tail skin. Measurements were averaged to provide a value for dorsal or ventral tail thickness for each animal.

RESULTS

I. Verification of Atrazine Exposure Concentrations

In the short term assay, there was not significant absorption of atrazine by the bioassay tray plastic (524.2 ± 685.6 ppb) or the substrate paper towels (700.9 ± 304.4 ppb). Concentrations of available atrazine were consistent with the prepared stock solution (794.5 ± 94.8 ppb) (Table 1).

In the long term assay, the plastic of the home boxes was not absorbing a significant amount of atrazine (251.9 ± 169.8 ppb); concentrations of atrazine were consistent with the prepared stock solution (241.1 ± 10.5 ppb). Home boxes with towels had reduced atrazine concentrations by approximately 60% (88.9 ± 21.5 ppb) following 1 week (Table 2). Towels were replaced once a week to account for this degradation so animals were exposed to desired levels of atrazine during chronic exposure studies.

Aim I—Effects of acute atrazine exposure on chemoinvestigation and vomeronasal organ (VNO) sensory neuronal activation.

I. Behavior

A. Nose-tapping response to chemosensory cues

In the first behavioral experiment, nose tapping was significantly higher on substrates containing a high dose of atrazine in subjects naïve to atrazine, suggesting that animals detected the atrazine (Figure 1; overall main effect of atrazine: $F(3, 93) = 8.150$, $P < 0.001$; within-subjects contrasts comparing nose-tapping on water versus atrazine $F(1, 31) = 6.923$, $P = 0.013$; versus atrazine +

non-gravid female alarm secretion $F(1,31) = 8.942, P = 0.005$). Contrary to expectation, nose tapping did not increase in the presence of non-gravid female alarm secretion when compared to water (Figure 1; $F(1, 31) = 2.509, P = 0.123$). Atrazine in combination with non-gravid female alarm secretion induced significantly more nose tapping than non-gravid female alarm secretion alone (Figure 1; $F(1, 31) = 14.681, P = 0.001$). Activity did not differ among treatment groups (Figure 2: $F(3, 93) = 1.795, P = 0.154$).

In the second experiment, animals were tested to determine if subjects could detect a range of doses of atrazine. None of the doses of atrazine elicited increased nose tapping (Figure 3; overall effect of dose: $F(3, 31) = 1.024, P = 0.390$; data log transformed to meet assumptions of normality and homogeneity of variances). In a second trial of the dose response study with an additional control group of males only exposed to water, males also did not increase nose-tapping on atrazine contaminated substrates (Figure 4: $F(3, 81) = 0.598, P = 0.618$). Also, males with previous atrazine exposure did not differ significantly from atrazine-naïve animals in mean number of nose taps when tested on substrates moistened with water.

The next experiment was conducted to determine if atrazine reduced the ability to detect a socially relevant chemosensory cue and a prey-derived chemosensory cue. Levels of nose tapping depended on the chemosensory cue (Figure 5, main effect of chemosensory cue: $F(5, 145) = 4.638, P = 0.001$). Animals nose tapped significantly more in the presence of prey cues, regardless of whether atrazine was present. Animals nose tapped more in the presence of

female body rinse, although this was not significant. However, there was no evidence that exposure to atrazine decreased nose tapping. Finally, activity was not different between groups ($F(5, 145) = 0.878, P = 0.497$) (data not shown).

In the nares occlusion experiment, there was a significant difference in nose tapping depending on chemosensory cue (Figure 6, main effect of chemosensory cue: $F(3, 90) = 15.560, P < 0.001$). Animals both with and without occluded nares nose tapped prey cues more than water ($F(1, 30) = 24.609, P < 0.001$). Despite the increased response to the prey cue in occluded animals, occlusion of the nares significantly reduced nose tapping in response to substrates moistened with chemosensory cues (Figure 6, main effect of occlusion: $F(1, 30) = 123.941, P = 0.002$). Atrazine by itself did not elicit increased nose tapping compared to the water control in either treatment group (7 ppb atrazine: $F(1, 30) = 1.125, P = 0.732$; 700 ppb atrazine: $F(1, 30) = 0.781, P = 0.796$).

B. Discrimination of atrazine versus water

Animals did not discriminate between water and atrazine at any dose (3.75 ppb, 75 ppb, 750 ppb) when given a choice between a substrate moistened with atrazine versus water for 2 hours. (Figure 7: not significantly different from 50% [2-tailed binomial test]: [3.75 ppb] $P = 0.281$, [75 ppb] $P = 0.720$, [750 ppb] $P = 0.281$). When checked after 12 hours of exposure, animals still did not discriminate between water and atrazine (Figure 7: not significantly different from 50% [2-tailed binomial test]: [3.75 ppb] $P = 0.856$, [75 ppb] $P = 0.720$, [750 ppb] $P = 0.720$).

II. Agmatine uptake after exposure to chemosensory stimuli

The number of AGB-immunoreactive cells in the VNO varied depending on the chemosensory cue applied (Figure 8, Figure 9: $F(3, 24) = 15.797$, $P < 0.001$, two-way ANOVA; data log transformed to correct for assumptions of homogeneity). Male animals exposed to mental gland extract had more AGB-IR cells than those exposed to water ($F(1, 24) = 39.753$, $P < 0.001$, two-way ANOVA with cue as fixed factor). Although there was no significant effect of atrazine on VNO sensory neuron activation ($F(1, 24) = 1.446$, $P = 0.241$), the interaction between atrazine and chemical cue was significant ($F(1, 24) = 6.193$, $P = 0.020$).

To better understand the significance of the interaction between atrazine and the chemical cue, the 2-way ANOVA was followed with a 1-way ANOVA and differences between the groups were compared with pairwise SNK post hoc tests. Post hoc tests revealed that the interaction was due primarily to increased AGB-IR in animals exposed to atrazine alone compared to animals exposed to water. There was no difference between animals exposed to MGE versus atrazine + MGE.

In a second study, neither atrazine nor earthworm wash increased agmatine uptake compared to water controls. There was no effect of atrazine (Figure 10: $F(1, 25) = 0.229$, $P = 0.636$), chemical cue ($F(1, 25) = 0.001$, $P = 0.975$) nor an interaction between atrazine and chemical cue ($F(1, 25) = 0.432$, $P = 0.517$). A subsequent study (Figure 11: main effect of chemosensory cue: F

(2, 9) = 8.702, $P = 0.013$) confirmed that earthworm wash does not activate cells of the VNO.

III. Hormone levels in response to atrazine exposure

There was no effect of chemosensory cue on corticosterone levels (Table 3: $F(1, 24) = 0.209$, $P = 0.652$), nor an interaction between chemosensory cue and atrazine ($F(1, 24) = 0.949$, $P = 0.340$). There was a significant effect of atrazine on corticosterone levels ($F(1, 24) = 7.423$, $P = 0.012$). However, a subsequent study found no effect of atrazine on plasma corticosterone (Table 4: $F(3, 24) = 0.633$, $P = 0.601$) or plasma testosterone ($F(3, 27) = 0.288$, $P = 0.833$) in animals exposed to atrazine.

Aim II—Effects of long-term atrazine exposure on chemoinvestigation, vomeronasal organ sensory neuron activation, feeding, plasma hormone levels, and tail gland morphology.

I. Behavior

A. Body Mass

Body mass did not change in any group over the three weeks of atrazine exposure ($F(1, 28) = 1.968$, $P = 0.172$).

B. Feeding

The mean number of flies eaten over all three nights (maximum = 30) was 15.43 ± 14.6 in animals exposed to atrazine and 15.53 ± 13.0 in controls. Long-term atrazine exposure did not alter feeding behavior; there was no difference in

the average number of flies consumed between the two groups ($F(1, 28) = 0.000$, $P = 0.984$). Feeding data was also not significantly different between groups (300 ppb versus 0 ppb) during each week of atrazine exposure (week 1: $F(1, 28) = 0.294$, $P = 0.592$; week 2: $F(1, 28) = 0.371$, $P = 0.548$; week 3: $F(1, 28) = 0.000$, $P = 0.994$).

C. Nose-tapping response to chemosensory cues

Animals nose tapped substrates moistened with prey cues and gravid female pheromones more than substrates moistened with water (Figure 12: effect of chemosensory cue: $F(2, 54) = 5.815$, $P = 0.005$, within-subjects contrasts comparing water to female body rinse and prey cue $P < 0.01$). There was no effect of atrazine exposure on nose tapping ($F(2, 54) = 0.524$, $P = 0.595$). Overall locomotor activity levels (data not shown) were not different depending on chemosensory cue ($F(2, 54) = 0.720$, $P = 0.491$) and there was no effect of atrazine ($F(2, 54) = 0.286$, $P = 0.753$).

II. Agmatine uptake in response to chemosensory stimuli

More sensory neurons were activated in the presence of mental gland extract when compared to water, regardless of previous atrazine exposure (Figure 13, Figure 14; overall effect of chemical cue: $F(1, 25) = 31.844$, $P < 0.001$). There was no effect of atrazine on AGB-immunoreactive cells (Figure 14; overall effect of atrazine treatment: $F(1, 25) = 0.009$, $P = 0.924$). There was no interaction between treatment and chemical cue (Figure 14: $F(1, 28) = 2.511$, $P = 0.126$).

III. Tail Gland Morphology

Long term exposure to atrazine-contaminated substrates did not reduce the tail gland dermal thickness dorsally (Figure 15: $F(1, 20) = 0.729$, $P = 0.404$) or ventrally ($F(1, 20) = 0.010$, $P = 0.921$). Gland size was similar to previously measured glands in non-atrazine exposed *P. shermani* (Largen and Woodley, submitted).

IV. Hormone levels

There was no significant effect of chemosensory cue on plasma corticosterone in animals with long term exposure to atrazine at 300 ppb (Table 3: $F(1, 15) = 2.470$, $P = 0.138$; data log transformed to correct for assumptions of homogeneity).

DISCUSSION

The role of atrazine as an info-disruptor in the amphibian *Plethodon shermani* was examined following both acute and chronic exposure. During acute exposure, where animals were exposed to atrazine for less than 24 hours, there was no effect on chemoinvestigation, VNO sensory neuron activation, or plasma hormone levels. Long term atrazine exposure (28 days) also did not disrupt nose tapping behavior, pheromone detection at the level of the VNO, pheromone production, or plasma hormone levels in salamanders. Therefore, atrazine does not appear to be acting as an odorant or an info-disruptor in *P. shermani*. These results will be discussed in more detail below.

Chemoinvestigation during acute atrazine exposure

Plethodontid salamanders nose tap to sample sensory cues in their environment, and increased nose tapping suggests increased interest in chemicals on the substrate. By placing their nares on the substrates, non-volatile substances are drawn into the VNO and detected by vomeronasal sensory neurons. Therefore, nose tapping can be used as an indicator of chemosensation.

In the first behavior study (Figure 1), animals increased nose tapping on atrazine-contaminated substrates, suggesting that atrazine was acting as an odorant and that the animals could detect the pesticide. In contrast to the initial behavioral study, a second experiment (Figure 5) found that although animals expressed increased nose tapping on prey cues, they did not chemoinvestigate atrazine-contaminated substrates more than water, suggesting atrazine was not

acting as an odorant or that animals were not motivated to respond to atrazine. Furthermore, atrazine did not dampen the nose tapping response to the prey cue. This suggests that animals would be able to successfully forage for prey if atrazine were present in the environment, because prey cues appear to be detected by the olfactory system, regardless of the presence of atrazine.

Additional behavioral studies also indicated that atrazine is not an odorant and also does not alter the ability to detect other chemosensory cues. For example, animals did not increase nose tapping in response to multiple doses of atrazine (Figure 3, 4). Also, in the nares occlusion study, although animals increased nose tapping in response to the wax worm macerate, neither occluded nor non-occluded animals increased nose tapping on atrazine at either high or low doses, indicating a lack of detection of atrazine on the substrate or a lack of motivation to chemoinvestigate atrazine (Figure 6). Interestingly, although occlusion of the nares reduced nose tapping significantly on all chemical cues, nares-occluded animals still nose tapped the wax worm macerate more than water, suggesting that the nares occlusion was not complete. Therefore, occlusion reduced, but did not eliminate, odor access to the nasal cavity. Possibly, some of the inert silicon gel was wiped off by the animal during the 12 hours between application and testing, or the seal over the nares was incomplete. Regardless of the success of the nares occlusion, the results of this study further indicate that atrazine is not acting as an odorant.

Animals did not behaviorally discriminate between substrates moistened with atrazine or water. Perhaps, animals were unable to detect atrazine in the

substrate and therefore had no preference or avoidance of it. Alternatively, atrazine may not have an advantage or disadvantage to the animal and therefore, animals were found randomly distributed between the two cues.

Overall, these behavioral results do not support the original hypotheses that atrazine is an odorant and/or it impairs the ability to detect other chemosensory cues. Other than the initial behavioral study, animals did not increase chemoinvestigation on substrates moistened with atrazine. In addition, atrazine did not dampen the ability to behaviorally respond to biologically relevant prey cues. The increased nose tapping response to atrazine in the first study may have been due to the fact that acetone was used in that experiment to help dissolve the atrazine. Perhaps animals were chemo-investigating the acetone and not the atrazine. Subsequent experiments did not use acetone to dissolve the atrazine.

Vomeronasal activation following acute atrazine exposure

Vomeronasal activation results were consistent with behavioral results following acute atrazine exposure, indicating that atrazine is not an odorant and it does not dampen the response of the VNO to chemosensory cues. In the first agmatine study (Figure 9), atrazine elicited a greater sensory neuron response than water, but a subsequent study (Figure 10) did not replicate this finding. Additionally, the VNO response to atrazine was significantly lower than the response to a known sensory neuronal stimulant, mental gland extract. Based on evidence in behavioral studies and supported by the second VNO study, I

conclude that atrazine is not acting as an odorant that can be detected by the VNO.

Responsiveness of the vomeronasal organ to the courtship pheromone, mental gland extract, was not impaired by acute atrazine exposure (Figure 9). The number of activated cells was similar in comparable studies of *P. shermani* (Wirsig-Wiechmann et al., 2002; Schubert et al., 2006; Schubert et al., 2008). Following acute exposure to atrazine, the ability of neurons in adult males to respond to the social cue mental gland extract is not impaired. This result indicates that atrazine in the environment should not reduce mating success as animals would still be able to detect cues from conspecifics.

It is difficult to conclude much about the effect of atrazine on detection of the food cue, earthworm wash, because earthworm wash did not activate VNO neurons (Figure 10). Although a previous pilot study (Nick Maddamma) indicated that earthworm wash activated sensory neurons of the VNO, a subsequent study indicated earthworm wash was not a reliable VNO stimulant (Figure 11). It was surprising that earthworm wash was not a VNO stimulus in *P. shermani* because previous work in garter snakes showed that earthworm wash elicited a VNO response (Jiang et al., 1990; Wang et al., 1993). Therefore, the ability of food cues, such as earthworm wash, to activate the VNO may be species specific, specific to the species of earthworm, or specific to the condition of the earthworm. Additional studies (data not shown) found that the prey cue waxworm macerate did not activate sensory neurons of the VNO in *P. shermani*, suggesting it is detected instead by the main olfactory epithelium.

Overall, the responsiveness of the VNO following acute atrazine exposure is in accord with behavioral results. They do not support the hypotheses that atrazine is an odorant and the ability to detect relevant social cues was not dampened. Sensory neuron activation in response to atrazine is much lower than the response to pheromones and was not consistent between studies.

Plasma hormone response following acute atrazine exposure

In a previous study (Hayes et al. 2006); male African clawed frogs had increased levels of plasma corticosterone following acute (4 day) exposure to a mixture of nine pesticides, including atrazine. The increased corticosterone levels in amphibians was linked to reduced growth and development (Hayes et al, 1995a, Hayes et al. 1995b, Hayes et al., 1997) as well as immunosuppression (Hayes et al., 1995b). In contrast to results from Hayes, I found that following acute atrazine exposure, animals had decreased plasma corticosterone levels compared to animals exposed to water (Table 3). However, in a second study of acute atrazine exposure on plasma hormone levels, there was no significant difference in treatment groups (Table 4). Thus, the initial results were unable to be replicated. The levels of plasma corticosterone in the first study were much higher than corticosterone levels than in animals found in the field (Woodley, unpublished) indicating that additional stressors and/or seasonal factors may have contributed to these high corticosterone levels in the animals in my study. Animals exposed to earthworm wash alone were also higher than other groups, potentially driving the significant difference. The increase plasma corticosterone

may be response to foraging cues. Overall, these results suggest that acute atrazine exposure does not elicit a stress response in *P. shermani* and therefore is not likely to compromise growth, development, or immune response.

Behavioral responses to long term atrazine exposure

Three weeks of exposure to atrazine did not dampen chemoinvestigation of prey cues or female pheromones. Both treatment groups had increased chemoinvestigation on chemosensory cues indicating long term atrazine exposure does not disrupt the ability of the olfactory system to detect chemosensory cues nor does it reduce behavioral responses to chemosensory cues. Chronic atrazine exposure did not impact body mass or feeding behavior, further indicating that animals were able to identify prey items, presumably via chemosensory cues. Animals were able to find prey items and consume the food; therefore atrazine does not affect foraging ability in *P. shermani*.

Long term atrazine exposure also did not increase overall locomotor activity; animals neither increased skittishness from exposure nor become more sluggish. There do not appear to be neurological impacts from long term atrazine exposure on behavior, contrasting with previous studies examining the impact of atrazine exposure on locomotor activity (Rohr and Crumrine, 2005; Rohr et al., 2003; Rohr and Palmer, 2005).

Vomer nasal response following long term atrazine exposure

Long term exposure to atrazine did not dampen the VNO response to the courtship pheromone, mental gland extract. In both treatment groups, more AGB-IR cells were found in animals exposed to male mental gland extract than in animals exposed to water. The number of activated cells was similar in comparable studies of *P. shermani* (Wirsig-Weichmann et al., 2002; Schubert et al., 2006; Schubert et al., 2008). This suggests that chronic exposure to atrazine would not reduce mating success via impaired pheromonal communication.

Tail gland responses to long term atrazine exposure

In previous studies in the male newt *N. viridescens*, the insecticide endosulfan reduced the size of pheromone secreting glands and reduced overall mating success (Park and Propper, 2002). In *P. shermani*, neither dorsal nor ventral tail gland sizes were reduced following long term exposure to atrazine. Dorsal tail glands are implicated in the secretion of anti-predator substance (Largen and Woodley, submitted), a copiously released proteinaceous substance that is used to deter predation (Hecker et al., 2003). Ventral tail glands may be used in scent marking of substrates and may contain pheromonal information about sex and reproductive condition (Largen and Woodley, submitted). In *P. shermani*, chronic atrazine exposure does not appear to alter the morphology of pheromone-producing tail glands; there was no reduction in tail gland size in animals chronically exposed to atrazine.

Plasma hormone levels in response to long term atrazine exposure

Circulating levels of corticosterone did not increase in animals chronically exposed to 300 ppb of atrazine, indicating that atrazine in the environment does not negatively impact hormonal regulation via corticosterone.

Conclusions

To conclude, I found no evidence that atrazine is an info-disruptor in *P. shermani*. Atrazine did not have consistent overall effects on chemoinvestigation, VNO sensory neuron activation, or plasma corticosterone levels in response to acute or chronic exposure. Feeding behavior was not impaired and tail gland morphology was not altered following long term exposure. Unlike previous aquatic model organisms used to examine the effects of atrazine, *Plethodon shermani* are terrestrial, which may have accounted for the lack of effect. Additionally, atrazine has been linked to developmental abnormalities, and these studies examined adult animals.

Although atrazine appears to be acting as an info-disruptor in several fish species, (Moore and Lower, 2001; Moore and Waring, 1998; Saglio and Trijasse, 1998), there is no evidence that atrazine is acting as an info-disruptor in amphibians. A study by Rohr et al. (submitted) found results consistent with our findings. *Bufo americanus* tadpoles were exposed to ecologically relevant concentrations (201 ppb) of atrazine for 4 days. There was no difference in responses to food, alarm chemicals, parasites, and predators between exposed and unexposed animals. Thus atrazine did not affect the ability of toads to respond to chemosensory cues. The varied results between species suggest that fish,

particularly via olfaction, are more sensitive to subtle atrazine exposure than amphibians.

Atrazine has received much attention as an endocrine disruptor (Hayes et al., 2002; Tavera-Mendoza et al., 2002) and is thought to potentially contribute to amphibian decline. However, several studies have failed to replicate Hayes' findings and have failed to determine a dose response relationship between atrazine and gonadal abnormalities (Carr et al., 2003; Hecker et al., 2003, Coady et al., 2004; Steeger, 2004). In contradiction to Hayes' work, Carr found that ecologically relevant concentrations of atrazine (1-25 ppb) did not influence metamorphosis or sex ratios in *X. laevis* nor growth, metamorphosis, or gonadal morphology in *Rana clamitans* (Carr et al, 2003; Coady et al., 2004). Additional work examining the role of atrazine on plasma steroid levels in *X. laevis* found that exposure did not affect aromatase activity, plasma hormone concentrations, nor germ cell development (Hecker et al., 2004; Hecker et al., 2005).

Additionally, in December 2007, the United States Environmental Protection Agency released a statement based on several studies that atrazine “does not adversely affect amphibian gonadal development, and believes there is no compelling reason to pursue additional testing of atrazine for amphibian gonadal effects”. These findings, combined with studies on info-disruption, suggest that atrazine is safer than originally thought and does not appear to disrupt endocrine function or chemical communication in amphibians.

While atrazine does not affect chemical communication in *Plethodon shermani*, this work was important in developing a model to examine info-

disruption in a terrestrial organism. Due to their heavy reliance on chemical communication, *P. shermani* are a good model system to examine info-disruption in a terrestrial species. Although my work with atrazine should not be generalized to include all info-disrupting chemicals or amphibians, this information suggests certain chemicals do not have harmful effects on adult amphibians. Often chemicals classified as endocrine disruptors have a greater impact on developing organisms and potential negative effects are minimal in developed adults. The multitudes of chemicals present in the environment are only marginally understood; their impact on wildlife and humans is even less well understood. Determining whether ubiquitous contaminants act as info-disruptors is important in understanding amphibian declines worldwide.

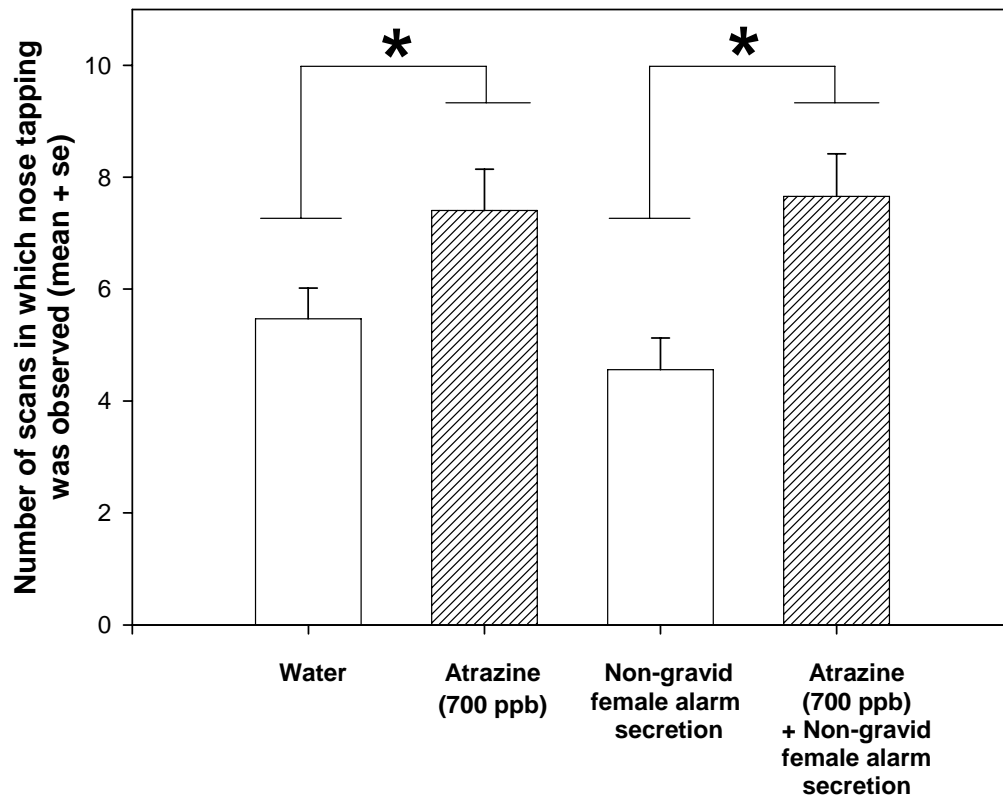


Figure 1. Levels of nose tapping in males ($n = 32$) upon exposure to substrates moistened with different chemosensory cues with or without atrazine. These males had not been previously exposed to atrazine. Nose tapping increased in the presence of atrazine-contaminated substrates. *significantly different as determined by within subjects contrasts ($P < 0.001$, repeated measures).

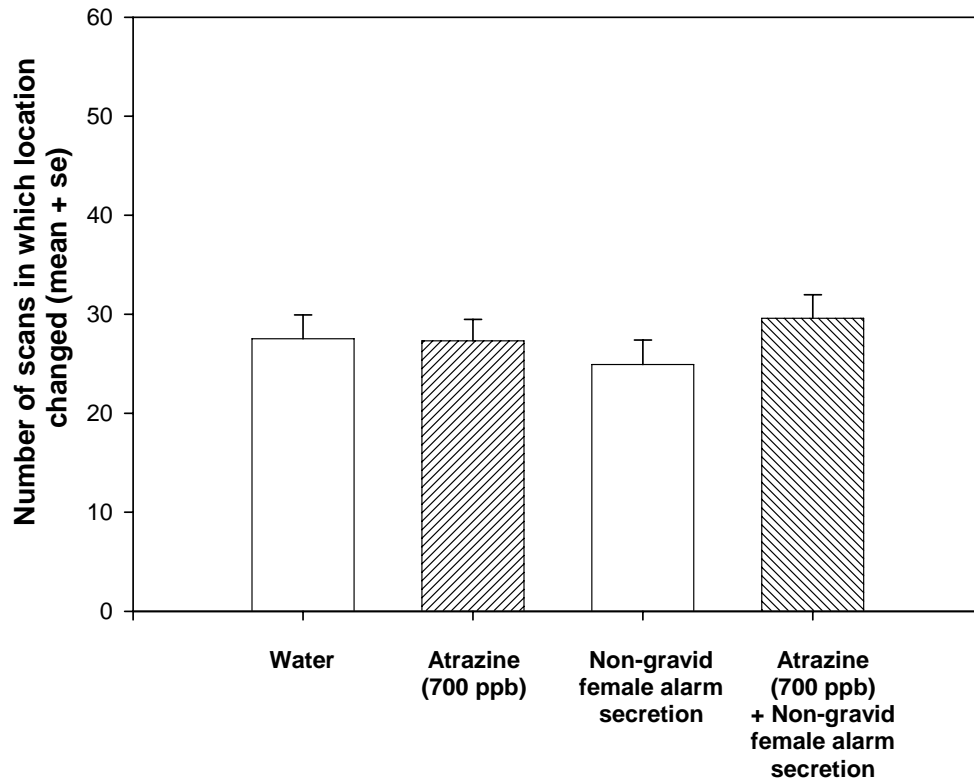


Figure 2. Levels of activity in males ($n = 32$) naïve to atrazine exposed to substrates moistened with different chemosensory cues with or without atrazine. Activity levels did not differ among treatment groups. ($P = 0.154$; repeated measures, within subjects contrasts).

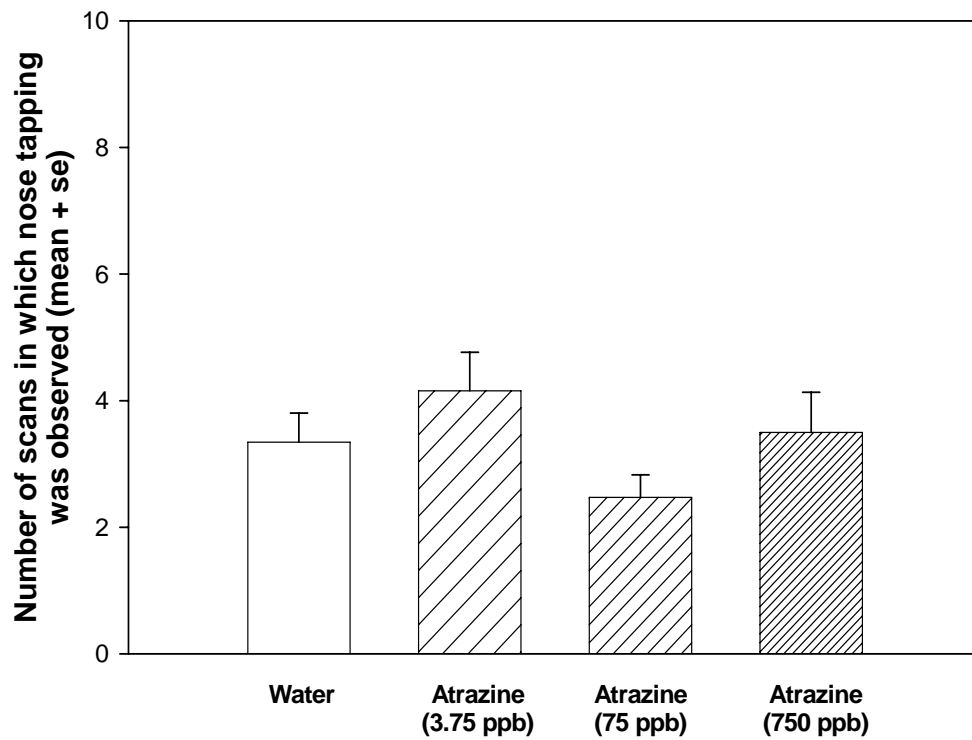


Figure 3. Levels of nose tapping in males ($n = 32$) exposed to substrates moistened with different doses of atrazine. Nose tapping did not increase on atrazine-contaminated substrates ($P = 0.390$; repeated measures, within subjects contrasts).

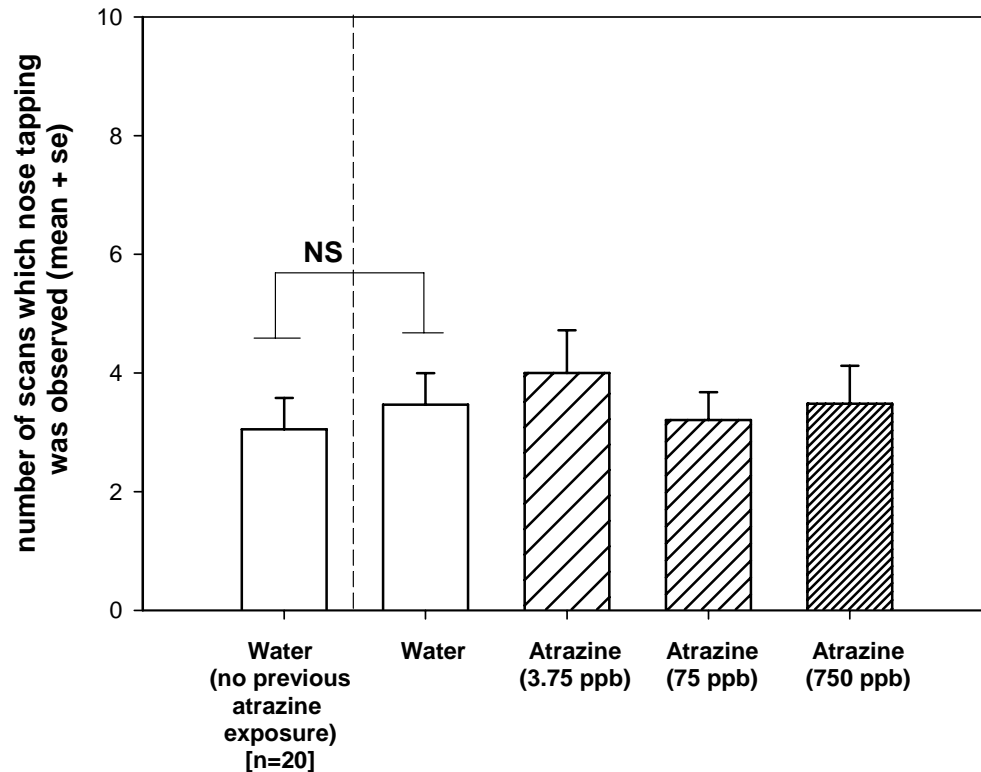


Figure 4. Levels of nose tapping in males ($n = 29$) previously exposed to atrazine in the presence of substrates moistened with different doses of atrazine. An additional group of 20 males with no previous atrazine exposure was included as an additional control; they were only tested in the presence of water to determine if brief exposure to atrazine in the repeated measures design altered overall nose tapping, even when tested on water. Nose tapping did not increase on atrazine-contaminated substrates ($P = 0.618$; repeated measures, within-subjects contrasts). Nose tapping on substrates moistened with water did not differ depending on whether animals had previous exposure to atrazine.

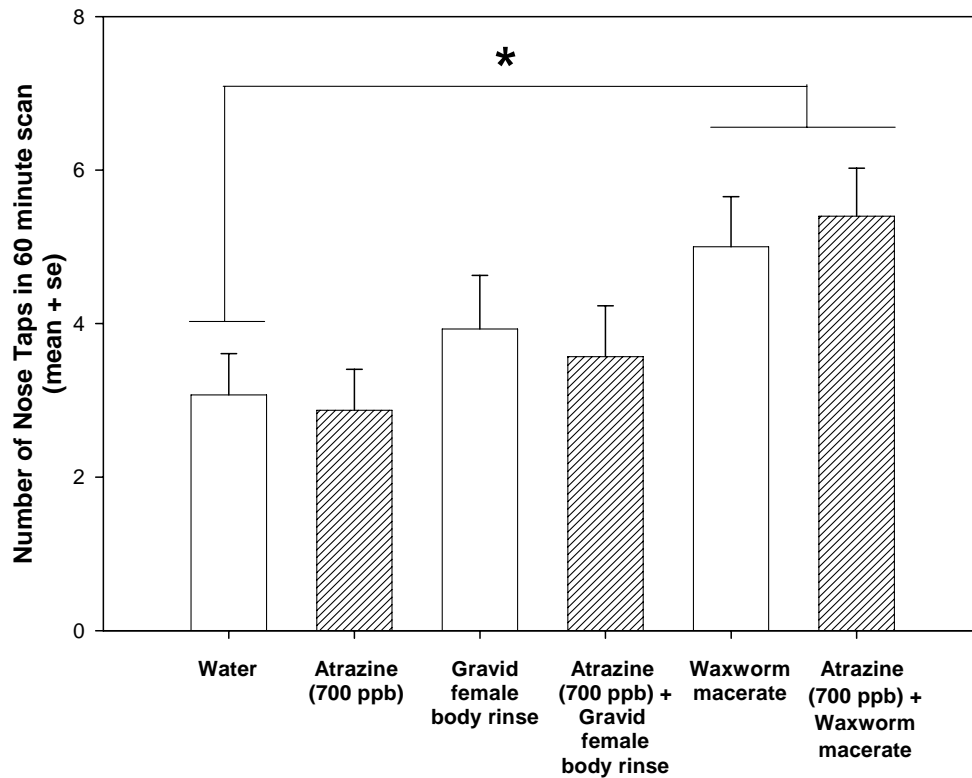


Figure 5. Levels of nose tapping in males ($n = 30$) exposed to substrates moistened with different chemosensory cues with or without atrazine. Animals nose tapped substrates moistened with prey cues significantly more than those moistened with water (* significantly different than water $P < 0.001$, within-subjects contrasts). Animals did not nose tap atrazine more than water and atrazine did not inhibit detection of prey cues.

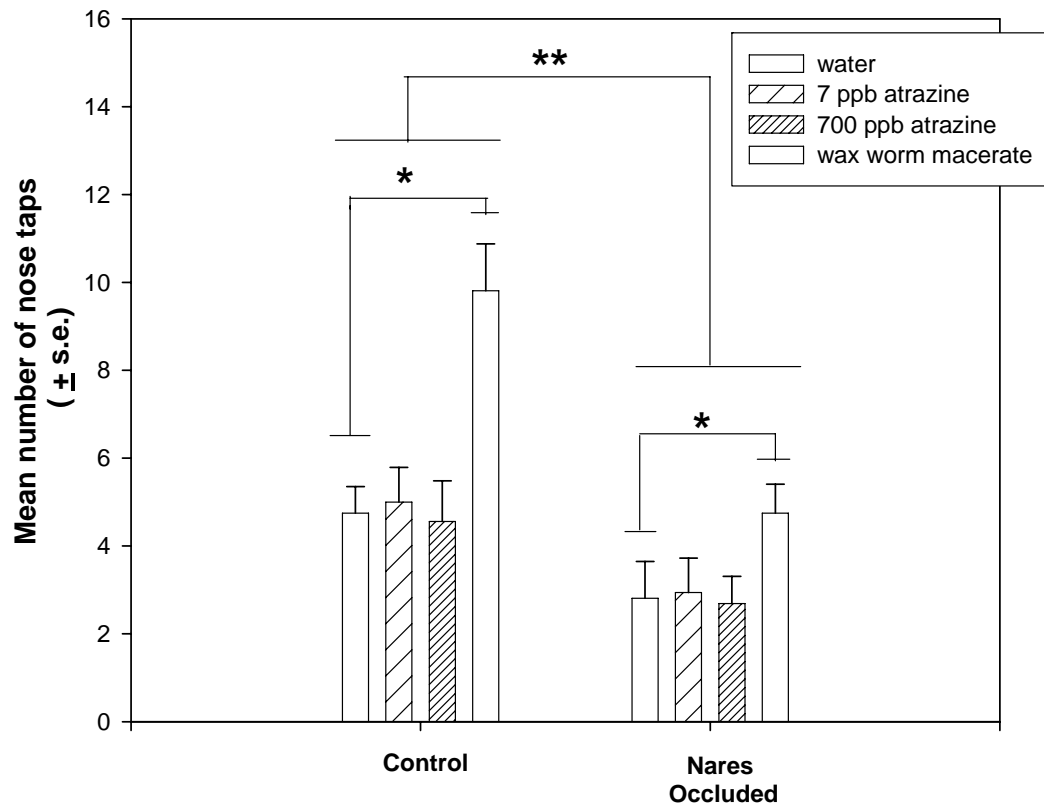


Figure 6. Females ($n = 16/\text{group}$) were tested to determine whether they could detect atrazine and if atrazine was detected by the olfactory system. Occlusion treatment significantly reduced, but did not eliminate, nose tapping (**significant main effect of nares occlusion, $P = 0.002$; repeated measures, between-subjects contrasts). Occluded and non-occluded animals demonstrated increased nose tapping on prey cue (*significantly different from water $P = 0.001$; repeated measures, within-subjects contrasts). Females did not demonstrate increased nose tapping on atrazine-contaminated substrates.

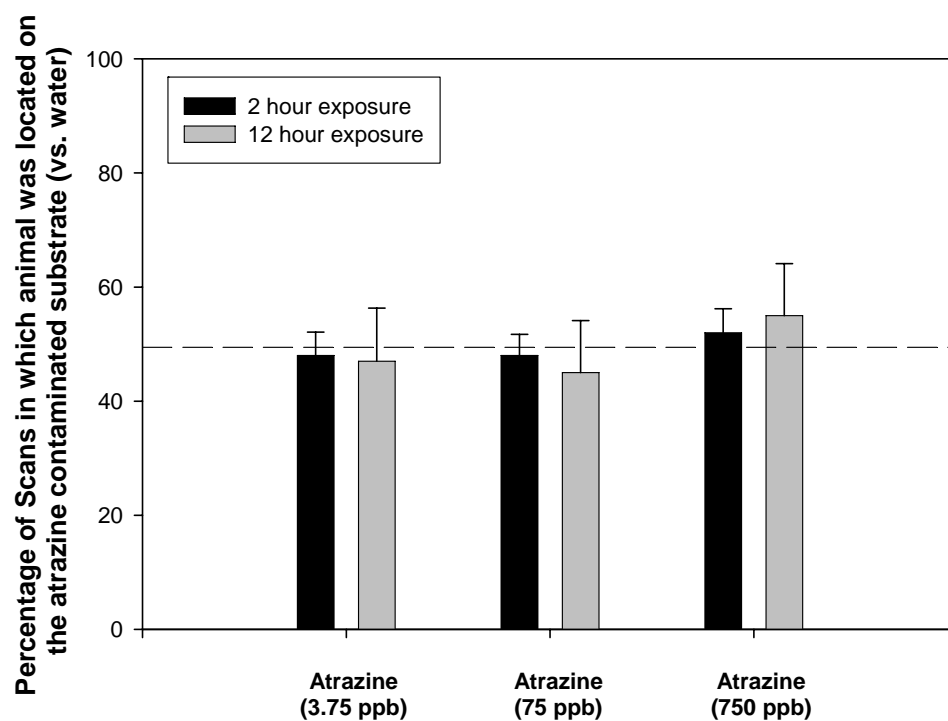


Figure 7. Males ($n = 31$) were tested for discrimination between water and each of 3 doses of atrazine during two hour tests. There was no significant preference discrimination between atrazine and water. After 12 hours of exposure, location was noted. There was no significant preference or avoidance for atrazine.

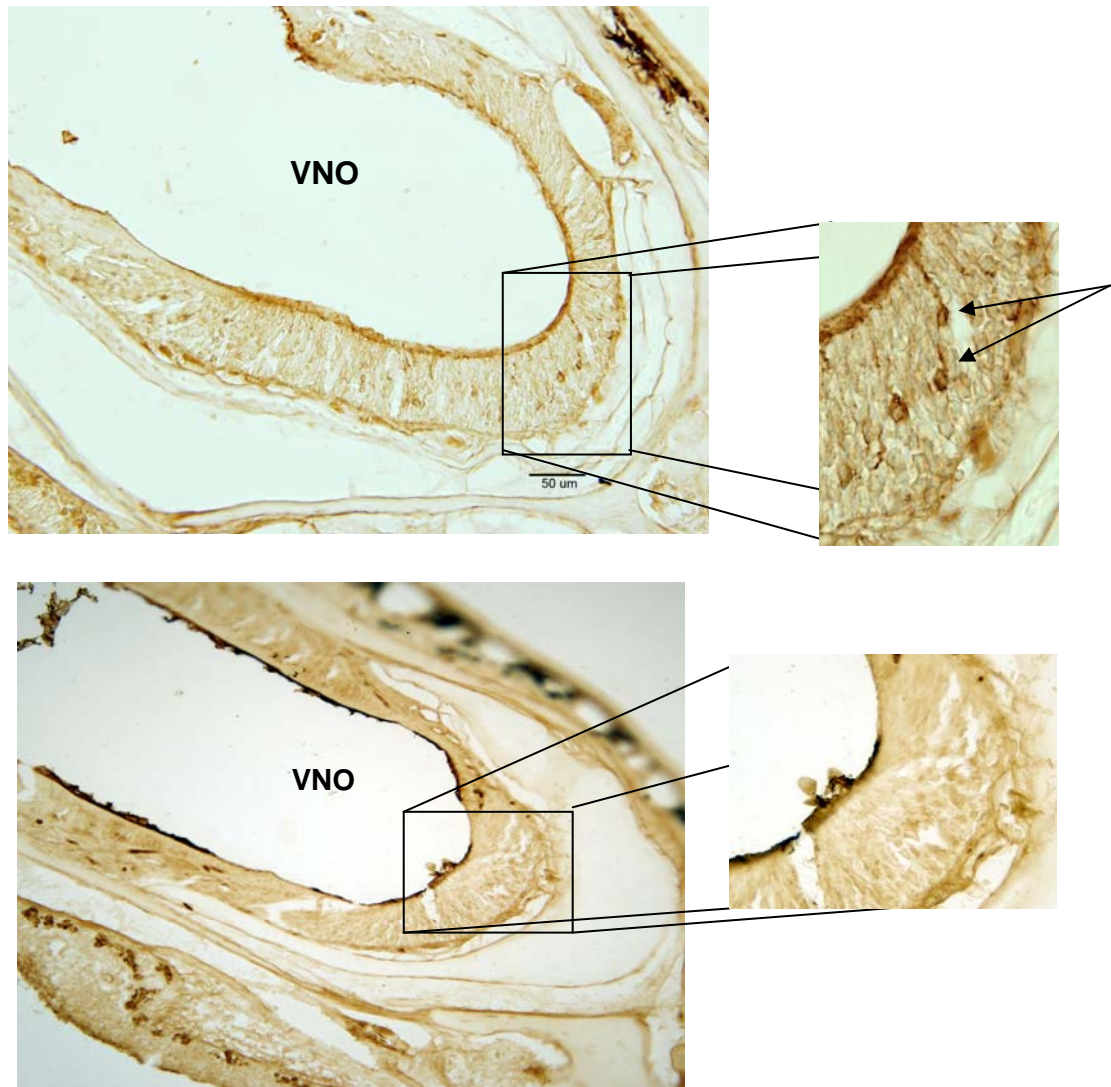


Figure 8. Representative photomicrographs of 20 μm coronal sections of the VNO. Top panel: A male that received mental gland extract, with or without atrazine, had many cells with AGB-IR. Arrows indicate AGB-IR cell bodies with dendrites. Bottom panel: A male that received atrazine or water with no mental gland extract had very few AGB-IR cells.

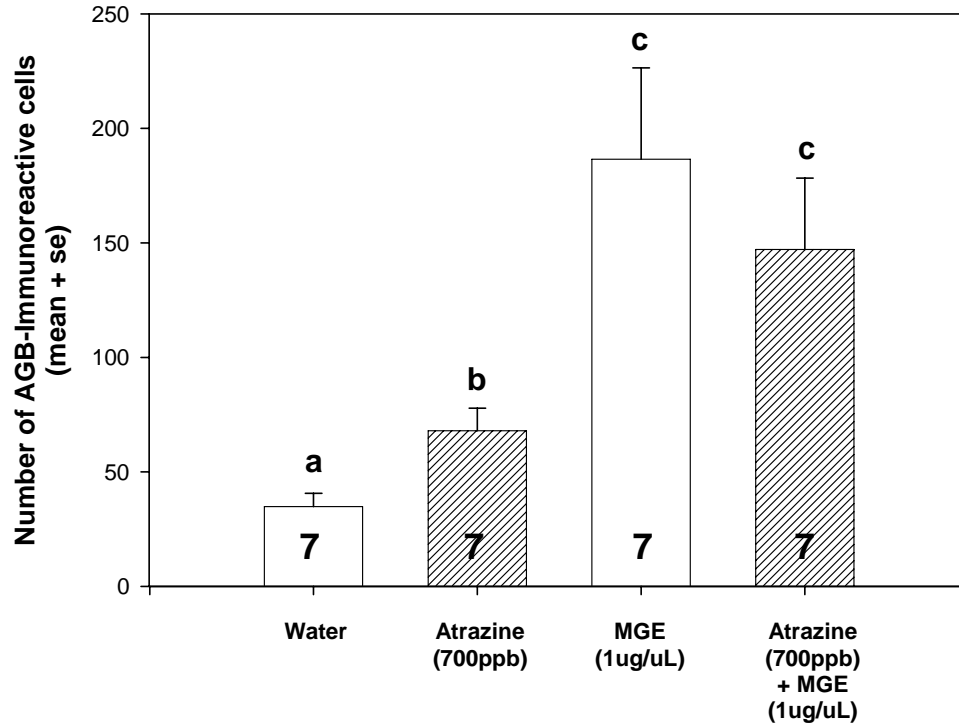


Figure 9. Number of AGB-IR cells in the VNO of males exposed to atrazine and/or mental gland extract. Groups that do not share a letter are significantly different from each other (1-way ANOVA; SNK post hoc comparison tests). Sample sizes indicated in bars.

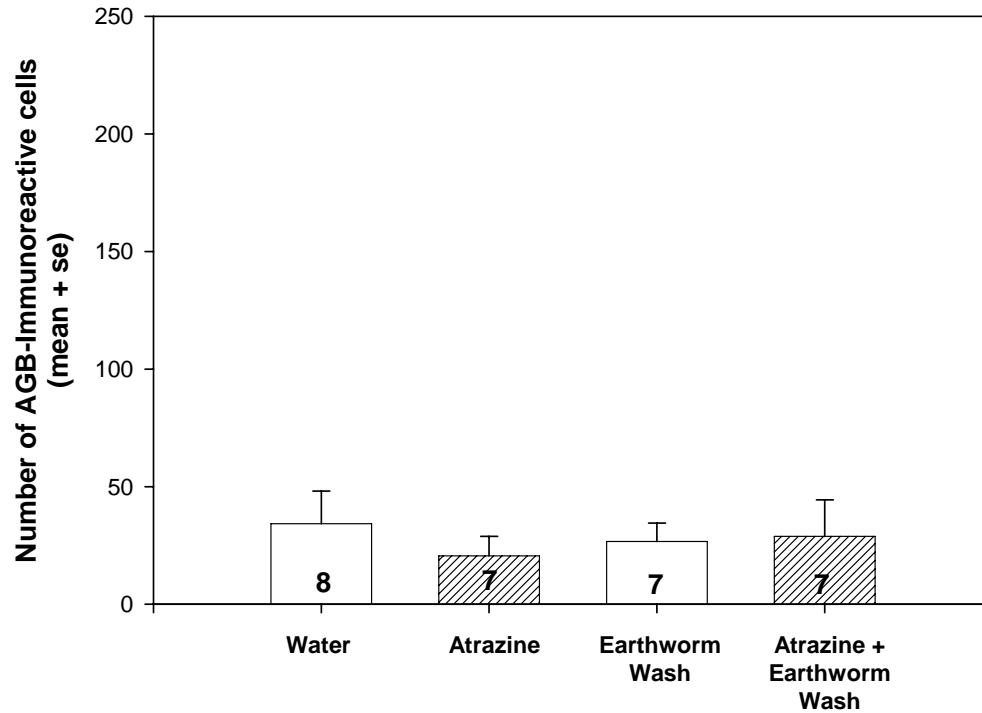


Figure 10. Number of AGB-IR cells in the VNO of males exposed to atrazine and/or earthworm wash 45 minutes before sacrifice. Groups were not significantly different from each other ($P = 0.876$; 2-way ANOVA). Sample sizes indicated in bars.

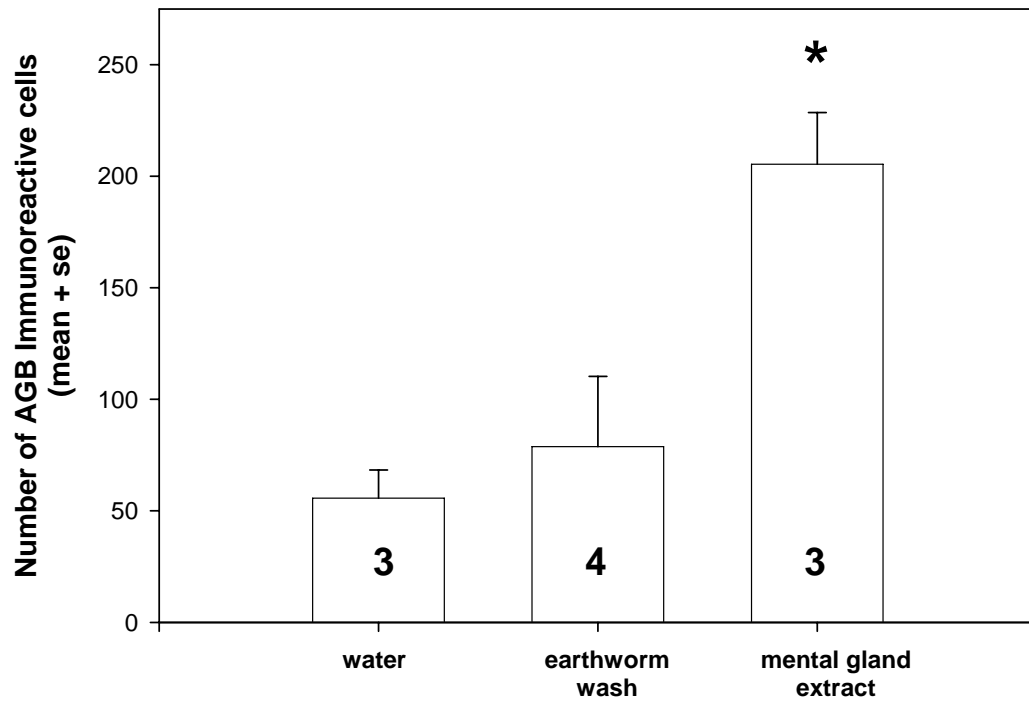


Figure 11. Number of AGB-IR cells in the VNO of males exposed to chemosensory cues. Earthworm wash did not activate cells of the VNO. Mental gland extract activated more cells of the VNO relative to water and earthworm wash (*significantly different than water as determined by 1-way ANOVA, $P = 0.013$). Sample sizes indicated in bars.

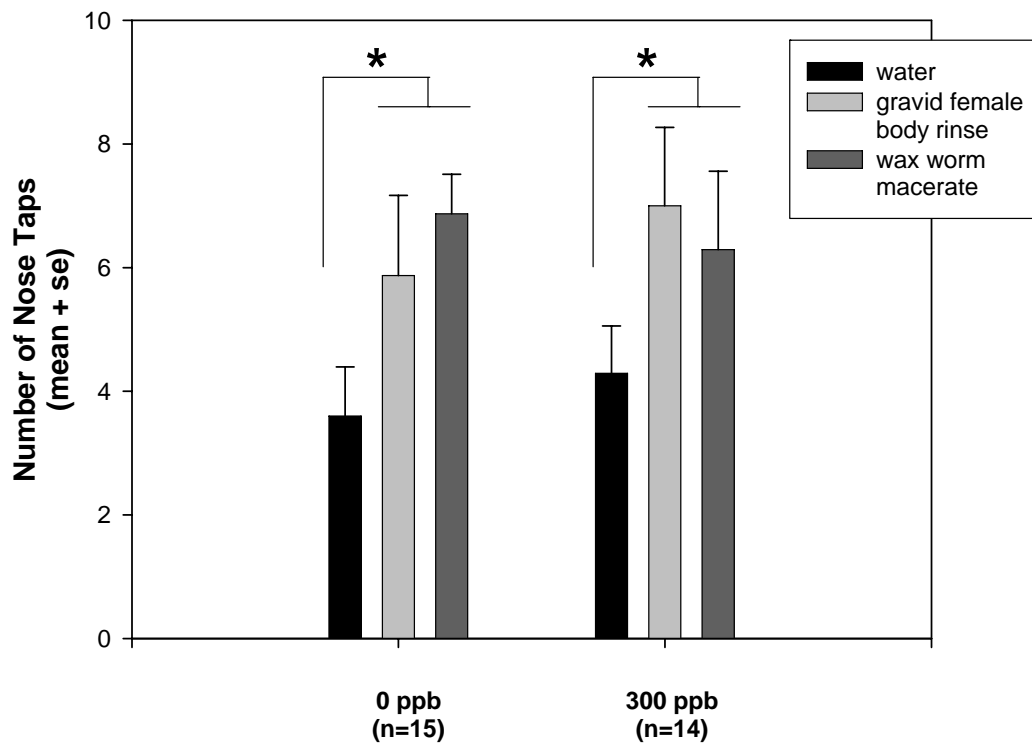


Figure 12. Levels of nose tapping in response to substrates moistened with different chemosensory cues in animals after twenty-eight days of exposure to atrazine. All animals nose tapped gravid female body rinse and wax worm macerate significantly more than water (*significantly different from water; repeated measures within-subjects contrasts; $P = 0.01$). There was no effect of atrazine exposure.

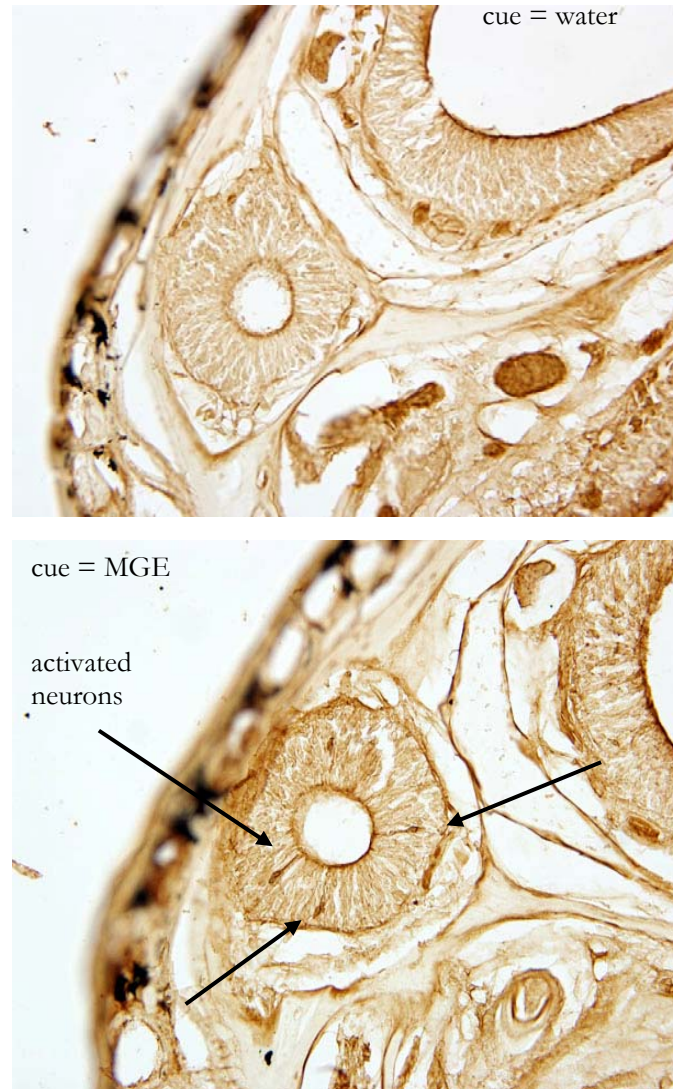


Figure 13. Representative photomicrographs of 20 µm coronal sections of the VNO. Top panel: A male that received water had very few activated AGB-IR cells. Bottom panel: A male that received mental gland extract had many cells with AGB-IR. Arrows indicate the AGB-IR cell bodies with dendrites.

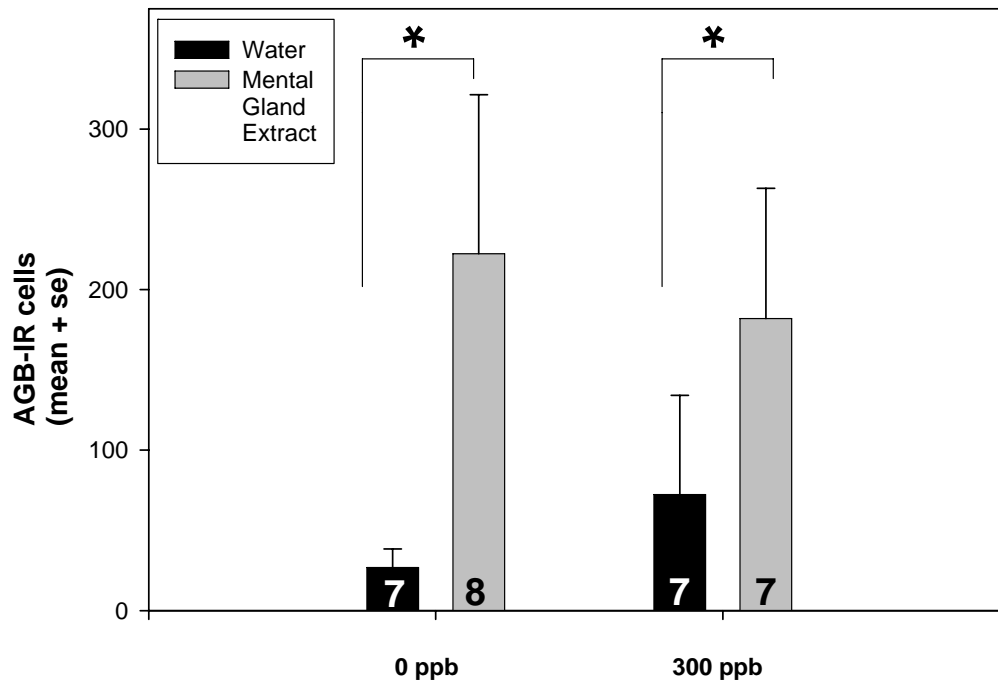


Figure 14. Number of AGB-IR cells in the VNO after exposure to water or mental gland extract in males chronically exposed to atrazine or water control. Exposure to mental gland extract increased the number of AGB-IR cells regardless of previous exposure to atrazine (*significantly different from water, 2-way ANOVA; $P < 0.001$).

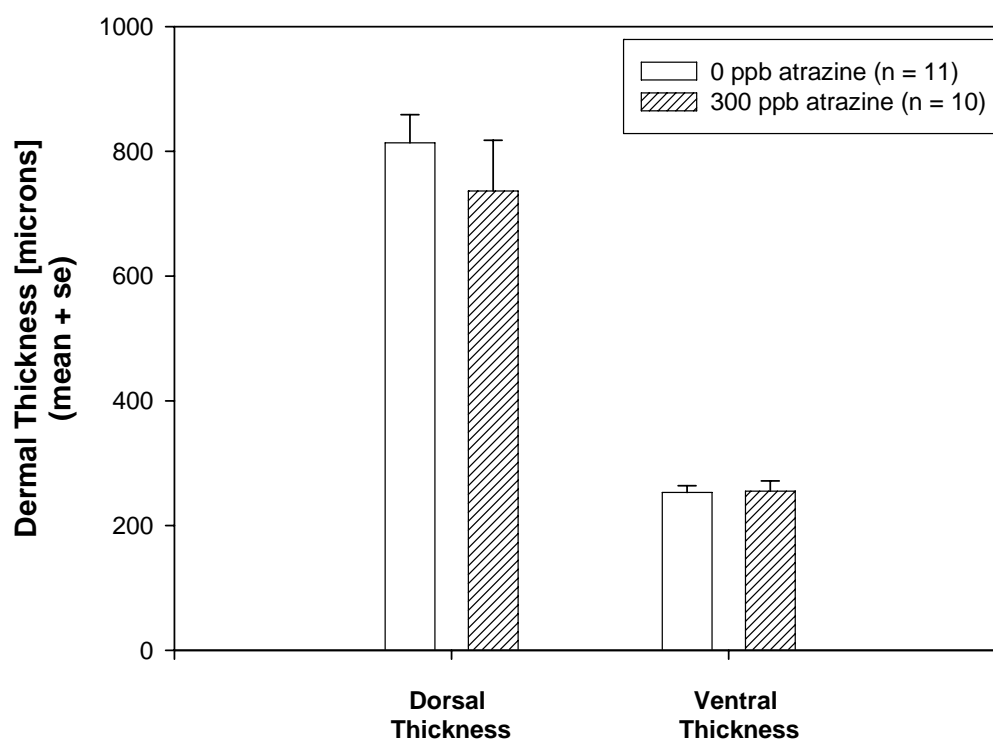


Figure 15. Long term exposure to atrazine does not significantly alter dermal thickness of dorsal ($P = 0.404$) or ventral ($P = 0.921$) tail glands as determined by 1-way ANOVA.

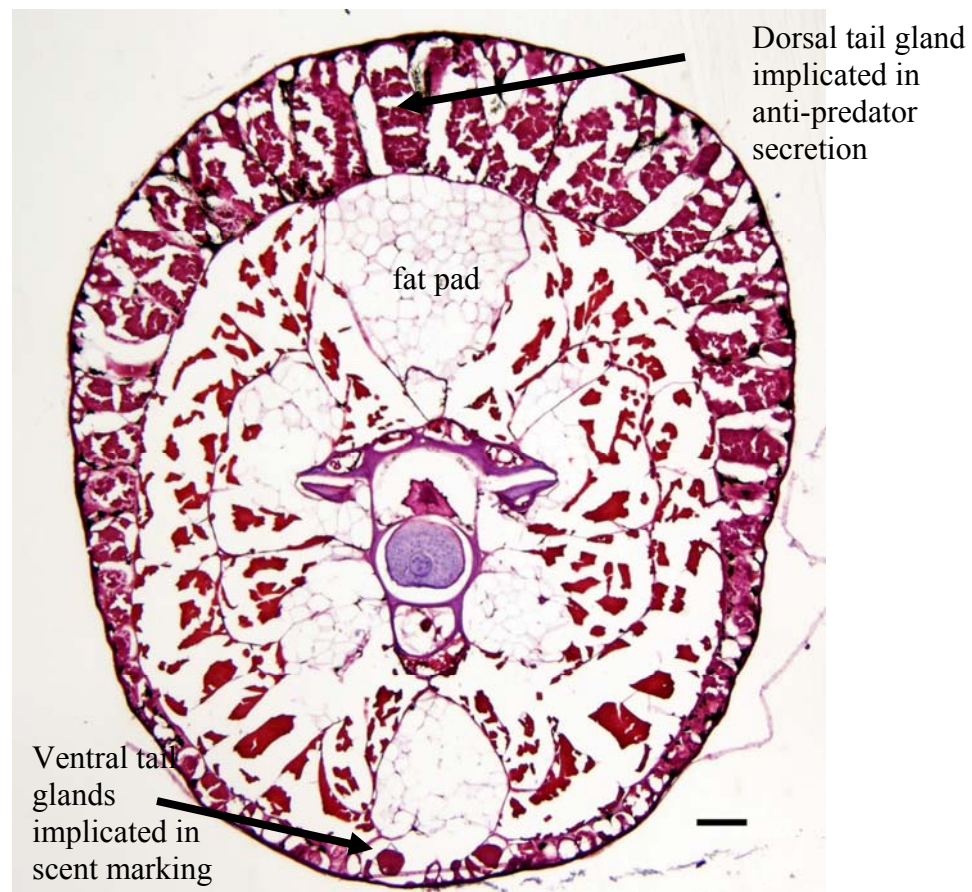


Figure 16. Representative photomicrographs at 30 μm coronal sections of tail morphology. Scale bar = 0.30 mm.

Treatment	Atrazine concentration (ppb)
ppb added (n = 2)	794.5 \pm 94.8
1 hour exposure without towel (n = 3)	524.2 \pm 685.6
1 hour exposure with towel (n = 3)	700.9 \pm 304.4

Table 1. Atrazine concentrations following 1 hour incubation in bioassay trays used during chemoinvestigation studies. Towels used during the study do not absorb atrazine; animals are presented with the expected concentration of atrazine during the 1 hour exposure.

Treatment	Atrazine concentration (ppb)
ppb added (n = 2)	241.1 \pm 10.5
7 day exposure without towel (n = 3)	251.9 \pm 169.8
7 day exposure with towel (n = 3)	88.9 \pm 21.5

Table 2. Atrazine concentrations following 7 day incubation in home boxes used to house animals during long term atrazine exposure. Towels used during the study do absorb atrazine; there was less available atrazine on the substrate. Following one week of exposure, the concentration of atrazine is reduced by approximately 60%, but changing towels on a weekly basis enables the chronic exposure concentrations to remain near the desired dose.

Treatment	Corticosterone (ng/ml)
Water	79.27 \pm 15.15
Atrazine	49.47 \pm 4.64
Earthworm Wash	103.63 \pm 29.99
Earthworm Wash + Atrazine	40.67 \pm 9.35

Table 3. Plasma corticosterone levels (mean \pm sem) in animals (7 per group) exposed to a chemosensory cue for 45 minutes prior to sacrifice. All animals were acutely exposed to atrazine during behavior studies previously. There was no significant effect of chemical cue (2-way ANOVA, $P = 0.652$) or atrazine (2-way ANOVA, $P = 0.340$) but there was an interaction between chemical cue and atrazine (2-way ANOVA, $P = 0.012$).

Treatment	Corticosterone (ng/ml)	Testosterone (ng/ml)
Water	60.19 \pm 5.26	53.04 \pm 22.47
Atrazine	62.71 \pm 10.31	69.51 \pm 28.33
Mental Gland Extract	68.67 \pm 10.07	63.60 \pm 21.67
Mental Gland Extract + Atrazine	52.81 \pm 6.10	42.81 \pm 12.32

Table 4. Plasma hormone levels (mean \pm sem) in animals (7 per group) exposed to a chemosensory cue for 45 minutes prior to sacrifice. All animals were naïve to atrazine prior to exposure. There was no effect of chemical cue or atrazine exposure on corticosterone levels ($P = 0.633$, 2-way ANOVA) or T levels ($P = 0.833$, 2-way ANOVA).

Treatment	Corticosterone (ng/ml)
0 ppb Atrazine	144.86 \pm 27.80
300 ppb Atrazine	90.73 \pm 10.14

Table 5. There was no significant difference between treatment groups (P = 0.138, One-way ANOVA) in plasma corticosterone levels.

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